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INNOVATIVE BIOREACTOR DEVELOPMENT FOR METHANOTROPHIC BIODEGRADATION OF TRICHLOROETHYLENE

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The objective of this work was to develop an innovative bench-scale two-stage bioreactor for biodegradation of trichloroethylene (TCE) based on a methanotrophic (copper-tolerant mutant of Methylosinus trichosporium OB3b) microbiological system. Methanotrophic cometabolism of TCE was enhanced by separating cell growth and solub methane monooxygenase (sMMO) production and recovery, from TCE contact and degradation. The bioreactor consists of a continuous stirred tank reactor (CSTR), series of four plug-flow reactor columns and a dewatering system. The reactor was pressurized to increase mass transfer of methane and oxygen to the cells.
The reactor was successfully operated at TCE feed concentrations ranging from 0.2 m to 20 mg/L. Degradation efficiencies were 99.5 percent to 70 percent, consecutive The addition of formate enhanced and stabilized reactor performance of degradation TCE at 10 mg/L. A preliminary process design for construction of a 0.5 gpm pilot-scale system was developed from bench-scale results.

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PREFACE

This report summarizes research activities conducted by the Oak Ridge National Laboratory (ORNL) and the Center for Environmental Biotechnology (CEB) of the University of Tennessee (via subcontract to ORNL) for Armstrong Laboratory through Military Interdepartmental Purchase Request (MIPR) No. N91-84, "Treatment of Chlorinated Organic Compounds with Aboveground Bioreactors," during the performance period from July 26, 1991 through September 30, 1992. The second-year performance period of October 1, 1992 through December 31, 1993 was conducted under MIPR No. N92-63.

The work activities summarized in this report have been conducted by the following researchers: Anthony V. Palumbo (Environmental Sciences Division, ORNL) and Terrence R. Donaldson (Chemical Technology Division, ORNL); Janet M. Strong-Gunderson (Postdoctoral Fellow, Oak Ridge Associated Universities); John L. Bowman (Research Associate, CEB); Michael F. Tschantz (graduate student, Department of Chemical Engineering and CEB); Frederick A. Evans (undergraduate student, Department of Chemical Engineering); Paul R. Bienkowski (Department of Chemical Engineering and CEB) and Gary S. Sayler (Department of Microbiology and Graduate Program in Ecology, and CEB). Susan L. Bergman (ESD/ORNL) provided technical assistance. The Project Manager was Stephen E. Herbes (ESD/ORNL). Richard S. Hanson of the University of Minnesota served as consultant during the work through subcontract to ORNL. The Project Officer was Alison Thomas (Armstrong Laboratory). Jim Spain (Armstrong Laboratory) reviewed the work plans and provided technical input during the progress of the work. The final report was typed by Kim Y. Henley (ESD/ORNL).

All or parts of Sections 2, 3, and 4 of this report have been modified as stand-alone documents and have been submitted individually to journals for publication.

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EXECUTIVE SUMMARY

A. OBJECTIVE

The overall goal of this work was to demonstrate and enhance the effectiveness of microbiological systems employing co-metabolic techniques to destroy trichloroethylene (TCE) in groundwater. Specific objectives were:

1. To design and construct an innovative bench-scale two-stage bioreactor for biodegradation of TCE;
2. To enhance production and recovery of soluble methane monooxygenase (sMMO) activity in methanotrophic microorganisms during and after TCE biotreatment;
3. To operate the bench-scale two-stage bioreactor system to develop a data base of biodegradation of TCE for system optimization and process scaleup;
4. To determine the advantage of pulsed flow over steady-state operation through computer process simulation using the empirical Alvarez-Cohen model for TCE degradation;
5. To develop a preliminary process design for construction of a 0.5 gallon-per-minute pilot system.

B. BACKGROUND

Since the mid-1980's considerable attention has been directed toward application of methanotrophic (methane-utilizing) microorganisms for bioremediation of TCE. These microorganisms, which grow aerobically on methane, possess the capability of degrading TCE to nonhazardous products. The process is co-metabolic, i.e., the microorganisms do not derive any energetic advantage from degradation of the TCE. For several years researchers at Oak Ridge National Laboratory (ORNL) and the Center for Environmental Biotechnology (CEB) of the University of Tennessee have been involved in developing, optimizing, and testing bioremediation technologies based on co-metabolic microbial processes for degradation of TCE. Initial studies at ORNL, in the late 1980s, demonstrated the effectiveness of a bench-scale trickle-filter bioreactor, operated in a recycle mode, to substantially lower TCE concentrations in an influent stream. In parallel efforts at CEB, researchers demonstrated substantial degradation of a suite of chlorinated organic contaminants in saturated upward-flow bioreactors.

The work described in this report proceeded concurrently with a field demonstration of co-metabolic methanotroph-based and pseudomonad-based bioreactor technologies for the Department of Energy (DOE) Office of Environmental Restoration and Waste Management, Office of Technology Development. The work described in this report complemented the K-25 Site demonstration by providing a test of an alternate bioreactor design and evaluating the effects of alternate operating conditions (eg. the addition of formate and/or other compounds to overcome the inhibitory effects of TCE) that were beyond the scope of the DOE-sponsored demonstration.

C. SCOPE

This report encompasses work conducted jointly by ORNL and CEB during the period from July 26, 1991 through December 31, 1993.

Work conducted during the first year consisted of: development of an experimental Test Plan (Task 1), and a series of laboratory and bench-scale experiments to optimize co-metabolic microbial degradation of TCE (Task 2). Task 2 included: maximization of sMMO production (Subtask 2.1-a), optimization of sMMO recovery following TCE exposure (Subtask 2.1-b), and development and demonstration of a dual stage bioreactor system (Subtask 2.2).

Work in the second year of the project consisted of four additional tasks: (1) a series of laboratory experiments to increase sMMO activity during production, and sMMO recovery following exposure to TCE, in the bench-scale bioreactor (Task 3); (2) a series of operational runs of the bench-scale bioreactor system under differing sets of input parameters to evaluate the TCE-degrading performance characteristics of the bioreactor system (Task 4); (3) development of a process simulation of the bioreactor system (Task 5); and (4) design of a 0.5 gallon-per-minute pilot-scale system (Task 6).

D. METHODOLOGY

The bioreactor system developed in the study was designed and operated to enhance methanotrophic co-metabolism of TCE based on current molecular and biochemical knowledge of the requirements for TCE biodegradation. This enhancement was facilitated by separating growth and enzyme production/rejuvenation in an environment containing methane, from TCE degradation in a plug-flow methane-free environment. The separation eliminates competitive inhibition by methane for available sMMO, and allows sMMO pools to regenerate after contacting with TCE (and possibly becoming deactivated). The rate of TCE degradation is dependent on the sMMO concentration (which is proportional to biomass) and the toxicity effects associated with contact with TCE are lessened by increased biomass; therefore, washout of the biomass through the effluent is undesirable. A dewatering device that recycles a cell-rich stream to the bioreactor was implemented to keep biomass levels high, combating dilution effects due to the abiotic feed. To improve the mass transfer of methane and oxygen to the cells for enhanced cellular growth rates, the reactor was pressurized, increasing the liquid-phase solubility of the gases.

E. TEST DESCRIPTION

The bioreactor system consists of a continuous stirred recirculating tank reactor (CSTR), a series of four plug-flow reactor columns, and a dewatering system. All components of the system in contact with experimental fluids are composed of stainless steel, glass, teflon, or viton rubber, to minimize adhesion of TCE or biofilm to the bioreactor internal surfaces. The system has been pressure tested to 11.2 atm without leakage, but normal operating pressure is in the range of 1 to 4 atm. Methane and oxygen introduction into the CSTR occurs through two mass flow controllers. The gases are mixed in a manifold and enter the CSTR through a porous metal diffuser, located at the bottom of the CSTR. Fluid, comprised of media and cells, is pumped through stainless steel tubing from the CSTR to a vertical series of four 6.35 cm I.D. stainless steel pipe TCE contacting columns through

a variable speed metering lab pump. Valves allow control of flow from the CSTR to any configuration of the four columns. The TCE contacting columns are 17.8 cm, 25.4 cm, 27.9 cm, and 33.0 cm, in length (from uppermost to lowest columns), and are connected to one another in series by stainless steel tubing.

During operation synthetic TCE-contaminated water was pumped to a stainless steel storage tank under a 1.7-atm oxygen blanket for subsequent introduction to the bioreactor system at the top of the uppermost TCE-contacting column. To keep cells from escaping with the effluent stream from the CSTR, a tangential-flow dewatering device with feed-side recycle was used. Liquid samples were obtained periodically from the feed line, at the bottom of each the four plug-flow columns, from the CSTR, and from the effluent line via valved sampling ports into screw cap septum vials sealed with teflon-lined silicone seals. TCE levels were calculated by gas chromatographic analysis of vial headspace samples. Oxygen concentrations were monitored as percentage of saturation of oxygen from dissolved oxygen probes located in the CSTR and at the bottom of the fourth plug-flow column.

F. RESULTS

Initial flask tests conducted to define initial bioreactor operating conditions showed that dissolved methane levels of 7 mg/L are optimal for expression of maximum specific sMMO activity in OB3b. In the absence of methane sMMO activity declined rapidly (60 percent-98 percent over 4 days), but was maintained at a constant level over this period by addition of 20 mM formate, apparently by maintaining the intracellular NADH supply. Maintenance of dissolved oxygen levels of 2-5 mg/L were optimal for expression of maximum specific sMMO activity; above this range sMMO declined slowly (about 25 percent by 8 mg/L) but specific growth declined precipitously (>95 percent reduction by 8 mg/L). Nitrate concentrations between 2 mM and 100 mM, phosphate levels of 2 mM to 25 mM, and iron and magnesium levels of greater than 30 μM, were found optimal for both OB3b growth and sMMO expression. Several vitamins (B-12, d-biotin, and pyridoxine) stimulated specific sMMO activity up to 60 percent at low μg/L levels, but suppressed sMMO expression at higher concentrations.

Flask tests conducted to optimize recovery of sMMO demonstrated that exposure of OB3b to 1 mg/L to 2 mg/L TCE caused more rapid loss of sMMO activity than did absence of methane alone, indicating that TCE toxicity to cell metabolic systems occurs at exposure to levels as low as 1 mg/L. Addition of formate caused an increase in specific sMMO activity in cells following exposure to TCE. The observed acceleration of sMMO recovery in the presence of formate may be due to the cells' use of formate as a carbon and energy source that is not dependent upon the presence of sMMO.

In bioreactor runs at feed and recycle flow rates of 2 mL/min and 10 mL/min respectively, the bioreactor system demonstrated effective degradation of TCE in the single-pass operating mode (i.e., without intermediate cell addition during the TCE contact phase) with a 4-hour retention time. The extend of degradation declined with increasing TCE concentrations in the feed, from 0.2 mg/L (>99.5 percent degradation) to 1 mg/L (94.5 percent), 3 mg/L (83.9 percent), 10 mg/L (83.4 percent), and 20 mg/L (70 percent-80 percent). Removal efficiencies of TCE were higher during "cross-flow" operation (i.e., when fresh cells were added between contact columns) than in single-pass operation: at an influent concentration of 1 mg/L TCE was reduced to less than 5 μg/L in the effluent (i.e., 99.5 percent degradation). The cross-flow mode demonstrated higher removals than the single-pass mode at 3 mg/L (91.3 percent degradation) and 10 mg/L (93.7 percent) as well. In each test effluent

concentrations in the cross-flow mode were reduced at least 50 percent below those measured in single-pass operation. This substantial reduction in effluent concentration is more significant from an application perspective than is percent removal.

Reduction of the extent of TCE removal (indicative of TCE toxicity to the microorganisms) was observed at feed concentrations of 10 mg/L or greater; the level of sMMO inhibition appeared to be related to the amount of TCE degraded. Addition of 1 mM formate enhanced and stabilized reactor performance in degradation of TCE fed at 10 mg/L, with sMMO rising back to a level comparable to its original value.

Results of experimental bioreactor runs were adequately represented by a mathematical simulation. An empirical constant (i.e., "transformation capacity") employed previously by Alvarez-Cohen and McCarty to account for TCE toxicity provided increased goodness of fit only at TCE concentrations greater than 20 mg/L; below this value the experimental data was fit equally well by Monod kinetics.

G. CONCLUSIONS

A recirculating pressurized two-stage bioreactor system has been developed at the bench scale and successfully operated for methanotrophic co-metabolic oxidation of TCE at feed solution concentrations ranging from 0.2 mg/L to 20 mg/L. The bioreactor system has demonstrated the effectiveness of separation of the co-metabolic TCE oxidation stage from that of biomass maintenance and growth.

The critical factor for maintaining efficient TCE degradation has been demonstrated to be the level of active microbial biomass that contacts TCE in a nonmethane competition mode. Maximum operating efficiency for TCE removal was not achieved during the bioreactor operational period due to mechanical failures of the system dewatering devices that allowed greater than 60 percent of the biomass to escape the system. However, even under these suboptimal conditions, treatment effectiveness was enhanced by extending the cell-TCE contacting time in additional TCE contacting columns.

System operating characteristics during cross-flow operation, in which fresh cells are introduced into the TCE-containing stream, improved TCE removal capacity significantly (i.e., reduced effluent TCE levels) over the performance obtained when cells were added only at the beginning of the contacting stage Performance enhancement increased at higher TCE concentrations. Results were consistent with mathematical modeling of the system that incorporated inactivation of TCE degradation by TCE. Existing mathematical models appear to be adequate for describing and predicting TCE removal kinetics in the two-stage bioreactor system.

Soluble methane monooxygenase (sMMO) activity was proved to be stable and robust, and inactivation of the enzyme was shown to be largely recoverable by addition of formate, presumably by enhancing synthesis of new enzyme.

H. RECOMMENDATIONS

Successful performance of the two-stage bioreactor technology in this project should lead into additional tests to demonstrate the effectiveness of the design in groundwater treatment applications. Because the biomass level was identified as a key variable, the present bioreactor unit should be modified to increase the effectiveness of biomass retention. Feedback controls should be installed on the system to ensure maintenance of operational parameters at optimal levels.

Operational tests should continue with the bench-scale unit presently available. Tests to evaluate effects of additional parameters should be completed in order to optimize operating conditions. The promising initial results of formate addition in enhancing recovery of sMMO following TCE treatment should be continued to optimize its effectiveness. In addition, preliminary results indicating similar effectiveness of other compounds should lead to tests in the bench-scale system. Test length should be increased to evaluate the long-term stability of the system. These tests should include using actual TCE-contaminated groundwater obtained from Air Force sites. A preliminary evaluation of the system's economics should be conducted to identify the operating variables that are most important in cost minimization.

Bench-scale results are already sufficiently promising to warrant plans to scale up the bioreactor design that was tested in this project for engineering evaluation. The 0.5 gallon-per-minute scale is recommended as the largest that could be developed on the basis of the performance of the present unit. However, additional tests, using the bench-scale unit, are needed to optimize flow rates and minimize reactor volumes. Additional engineering design would then be required prior to construction of a pilot unit to optimize reactor configuration.

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SECTION I

INTRODUCTION

A. OBJECTIVES

The overall goal of this work is to demonstrate and enhance the effectiveness of microbiological systems employing co-metabolic techniques to destroy trichloroethylene (TCE) in groundwater. Specific objectives are:

1. To design and construct an innovative bench-scale two-stage bioreactor for biodegradation of TCE;
2. To enhance production and recovery of soluble methane monooxygenase (sMMO) activity in methanotrophic microorganisms during and after TCE biotreatment;
3. To operate the bench-scale two-stage bioreactor system to develop a data base of biodegradation of TCE for system optimization and process scaleup;
4. To determine the advantage of pulsed flow over steady-state operation through computer process simulation using the empirical Alvarez-Cohen model for TCE degradation;
5. To develop a preliminary process design for construction of a 0.5 gallon-per-minute pilot system.

B. BACKGROUND

1. Statement of the Problem

The U. S. Air Force has identified over 700 sites which are contaminated with mixtures of chlorinated solvents, including TCE and chlorinated ethanes. Conventional "pump-and-treat" remediation strategies primarily involve transfer of contaminants between media, i. e. from groundwater to the atmosphere or onto activated carbon. Bioremediation, by potentially enabling the complete destruction of contaminants to minimally hazardous inorganic constituents, may significantly improve the environmental acceptability of Air Force groundwater remediation efforts, as well as reducing costs below those associated with conventional cleanup technologies.

2. Methanotrophic Biotreatment

Since the mid-1980's considerable attention has been directed toward application of methanotrophic (methane-utilizing) microorganisms toward bioremediation of TCE. These microorganisms, which grow aerobically on methane, possess the capability of degrading TCE to nonhazardous products. The process is co-metabolic, i.e. the microorganisms do not derive any energetic advantage from degradation of the TCE.

Researchers at Oak Ridge National Laboratory (ORNL) and at the Center for Environmental Biotechnology (CEB) of the University of Tennessee have for several years been involved in developing, optimizing, and testing bioremediation technologies based on co-metabolic microbial processes for degradation of TCE. Initial studies at ORNL, in the late 1980s, demonstrated the effectiveness of methanotrophic microorganisms in a bench-scale trickle-filter bioreactor, operated

in a recycle mode, to substantially lower TCE concentrations in an influent stream (Strandberg et al., 1989). Supplemental studies resulted in the first isolation of a methanotrophic strain capable of TCE biodegradation (Little et al., 1988) and in characterization of their substrate preferences and nutritional requirements (Eng et al., 1991; Palumbo et al., 1991). In parallel efforts at CEB, researchers demonstrated substantial degradation of a suite of chlorinated organic contaminants in saturated upward-gradient bioreactors (Phelps et al., 1990, 1991).

3. Biotreatment Demonstration at the Oak Ridge K-25 Site

The work described in this report proceeded concurrently with a field demonstration of co-metabolic methanotroph-based and pseudomonad-based bioreactor technologies for the DOE Office of Environmental Restoration and Waste Management, Office of Technology Development. The demonstration, which was conducted at the Oak Ridge K-25 Site, consisted of operation of a 0.5 gallon-per-minute methanotroph-based trickle-filter unit, followed by a second one-month period of operation of a bench-scale pseudomonad-based unit using the same input stream. The methanotroph trickle-filter unit was loaned to ORNL from Armstrong Laboratory following a similar demonstration at an Air Force Base by Battelle-Columbus, Inc. under contract to the Armstrong Laboratory. The work described in this report was specifically designed to complement the K-25 Site demonstration by providing a test of an alternate bioreactor design and evaluating the effects of alternate operating conditions (eg. the addition of formate and/or other compounds to overcome the inhibitory effects of TCE) that were beyond the scope of the DOE-sponsored demonstration.

Operation of the K-25 Site demonstration bioreactor unit was initiated in September 1991 using steam stripping pretreatment. Following shutdown of the system in November 1991, the bioreactor was restarted in May 1992. The methanotrophic bioreactor unit was operated intermittently from June through August 1992 using an air oxidation pretreatment system, and then from April through July 1993 using the steam stripper pretreatment system. Operational results were equivocal: methane consumption exceeded 90 percent of the influent during most of both operating periods, indicating a thriving methanotrophic population in the bioreactors, but calculated TCE degradation rates were variable due to problems in accurate measurement of TCE concentrations and gas flows in the off-gas stream. Results are presently being compiled and interpreted in the final project report.

Throughout the demonstration project period ORNL staff collaborated with researchers at Envirogen, Inc. (Lawrenceville, N. J.) to develop and evaluate the applicability of several innovative co-metabolic pseudomonad-based bioreactor systems. Bench-scale tests were performed by Envirogen during 1991-1992 under subcontract to ORNL to evaluate the ability of the microorganisms to degrade TCE in the presence of the complex mixture of both chlorinated and aromatic hydrocarbons found in the K-25 Site groundwater. This work led to an agreement by Envirogen and Martin Marietta Energy Systems (the operating contractor of ORNL for DOE) to enter into a Cooperative R&D Agreement (CRADA) to demonstrate jointly Envirogen's proprietary pseudomonad-based bench-scale bioreactor unit at the demonstration site.

The Envirogen unit was installed in a van trailer at the site in July 1993, and startup/shakedown testing, using the steam stripper condensate as input to the unit, was conducted through September 1993. Instrument and hardware problems and apparent toxicity of the condensate

to the microorganisms limited operation to several periods of 3-4 days each. Results are presently being evaluated; initial data indicate that high removal rates of the influent TCE occurred at least intermittently.

C. SCOPE

This report encompasses work conducted jointly by ORNL and CEB during the period from July 26, 1991 through December 31, 1993.

Work conducted during the first year consisted of two tasks which proceeded sequentially. Task 1 consisted of development of an experimental Test Plan. Task 2 consisted of a series of laboratory and bench-scale experiments to optimize co-metabolic microbial degradation of TCE. The experimental work conducted during the first year of effort consisted of two subtasks which proceeded in parallel:

Subtask 2.1. Maximization of soluble methane monooxygenase (sMMO) activity

(a) Maximization of sMMO production
(b) Optimization of sMMO recovery following TCE exposure

Subtask 2.2. Development and demonstration of a dual-stage bioreactor system

Work in the second year of the project consists of four additional tasks. Task 3 consisted of a series of laboratory experiments to maximize sMMO activity during production, and sMMO recovery following exposure to TCE, in the bench-scale bioreactor (i. e., extension of Subtask 2.1). Task 4 consisted of a series of operational runs of the bench-scale bioreactor system under differing sets of input parameters to evaluate the TCE-degrading performance characteristics of the bioreactor system (followup of Subtask 2.2). Task 5 consisted of development of a process simulation (i. e., a mathematical model) of the bioreactor system. Task 6 consisted of design of a pilot-scale system based on the set of optimal operating conditions defined by Tasks 3 and 4, and making use of the process simulation developed in Task 5.

Because mechanical problems in the bioreactor caused schedule slippage during the project, not all of the bioreactor runs initially planned in a factorial design for the second year of work (Task 4) to optimize operational conditions were conducted. Independent variables tested included: TCE inlet concentration; influent flow rate; presence/absence of formate; oxygen/methane concentrations. Reactor configurations tested included treatment through the four contactor columns in series with and without cell addition, and with cell addition between Columns 2 and 3. Because of operational problems with the cell separation device, the effect of different rates of cell wastage was not evaluated. Originally, it was planned to use the strain *Methylosinus trichosporium* OB3b (= ATCC 35069) in all experiments. Initial bioreactor experiments indicated the presence of a low level of copper ($\sim$ 1 μM) was interfering with sMMO activity of the strain. To alleviate this problem, a copper tolerant mutant of *Methylosinus trichosporium* OB3b was utilized in all bioreactor experiments. The mutant (designated PP358; obtained from Dr. George Georgiou, Dept. of Chemical Engineering, the University of Texas at Austin) (Phelps et al., 1992) is identical to the wild-type except that its ability to metabolize copper has been disabled (Fitch et al., 1993). Modeling studies (Task 5) compared once-through operation with the cross-flow mode actually employed in the final reactor

design, rather than a pulsed-flow system as initially planned. Modeling studies and process optimization (Task 6) were based on the operational data that were obtained, and thus may not necessarily represent the absolute optimal bioreactor operating conditions.

SECTION II

OPTIMIZATION OF SOLUBLE METHANE MONOOXYGENASE ACTIVITY

A. INTRODUCTION

Soluble methane monooxygenase (sMMO) and particulate methane monooxygenase (pMMO) are two different enzymes which carry out essentially the same function, however they coexist in certain methanotrophic strains. These enzymes have been extensively studied in *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* OB3b (Dalton 1992, Murrell 1992) but they are also found in *Methylosinus sporium* strain 5 (Brusseau et al., 1990; Pilkington and Dalton, 1991), *Methylocystis* sp. M (Uchiyama et al. 1992), *Methylomonas methanica* 68-1 (Koh et al., 1993), *Methylobacterium* sp. CRL-26 (Patel and Savas, 1987), and in various groundwater isolates (Bowman et al., 1993). Though widely distributed sMMO appears to be a strain-specific trait (Koh et al., 1993; Bowman et al., 1993) while pMMO is universal among all methanotrophs studied so far (Dalton 1992). It has been postulated that methanotrophs producing sMMO may have evolved in habitats which are regularly starved of copper (Prior and Dalton 1985). This cannot be supported empirically, however. sMMO and pMMO synthesis is regulated by copper availability. Copper suppresses the synthesis of sMMO while it promotes pMMO synthesis (Stanley et al., 1983).

TCE has become a major target for developing bioremediation processes using methanotrophs (Hazen, 1992; Semprini et al., 1992) owing to its importance as a relatively common, recalcitrant, environmental pollutant (Ensley, 1991) as well as its potential carcinogenic threat (Infante and Tsongas, 1979). The enzyme sMMO has been shown to co-oxidize TCE one- or two-orders of magnitude more efficiently than pMMO (DiSpirito et al., 1992) and other broad specificity monooxygenase and dioxygenase systems (Ensley, 1991). Soluble MMO can insert oxygen into alkanes, haloalkanes, alkenes, ethers, alicyclic, aromatic, and heterocyclic compounds. By comparison, pMMO has a significantly narrower substrate range. The mechanistic aspects of sMMO have been recently reviewed by Dalton (1992). Methods are available for specifically quantifying sMMO activity. For instance, sMMO can be assayed by its capacity to oxidize cyclohexane to cyclohexanol (Colby et al., 1977) or naphthalene to naphthol (Brusseau et al., 1990; Koh et al., 1993). These substrates cannot be oxidized by pMMO.

Recent interest in developing strategies for removal of chlorinated aliphatic contaminants from groundwater aquifers has led in a number of directions. A major path has led to the development of sMMO-based bioremediation systems. *In situ* systems involving the stimulation of an indigenous methanotrophic microflora to degrade TCE and other similar contaminants have been extensively studied (Hazen, 1992; Semprini et al., 1992). "Pump-and-treat" bioreactor-based strategies are still in development with relatively few field-scale systems having been implemented. Multistage, dispersed growth reactor systems have considerable potential for reaching the field-scale application level (Alvarez-Cohen and McCarty, 1991b; McFarland et al., 1992). These systems have the advantage that methanotrophs can be grown in one stage continually maintaining levels of sMMO activity and avoiding the problem of TCE toxicity. Exposure of cells to contaminants takes place in an adjacent stage with subsequent disposal of the cells.

A number of studies have attempted to define cultural conditions needed to obtain sMMO activity in methanotrophs and thus observe heightened TCE degradation. Most studies indicate that

copper availability is the most critical factor in obtaining sMMO activity (Oldenhuis et al., 1989; Tsien et al., 1989). Certain studies have focused on obtaining high rates of TCE degradation in resting cell assays. For instance the use of artificial electron donors such as formate enhance sMMO activity (Oldenhuis et al., 1989; Brusseau et al., 1990). Other factors such as the effects of methane competition on TCE degradation (Oldenhuis et al. 1990; Broholm et al., 1992) and TCE toxicity have also been closely studied (Alvarez-Cohen and McCarty, 1991a). Relatively few studies have attempted to determine exactly how sMMO activity can be maximized in methanotrophs during growth and then be maintained over extended periods. Studies by Park et al. (1992) revealed that certain nutrients, including CO_2, iron, nitrate, and phosphate were important in obtaining good growth rates and yields of the methanotroph *Methylosinus trichosporium* OB3b; these studies did not indicate clearly how sMMO activity may be optimized independent of growth rates.

As part of a larger effort involving the development of an innovative bioreactor system for sMMO-based TCE biotreatment, studies involving sMMO optimization in batch culture were undertaken. The factors critical for maximal, long term, and stable sMMO activity in the strain *Methylosinus trichosporium* OB3b was investigated. Factors studied included the supply of reducing equivalents and nutrients, and stimulation of sMMO synthesis with exogenous growth factors. This information is important if sMMO-based bioreactor systems are to be truly optimized for the efficient degradation of TCE and other halogenated aliphatic compounds. (Some of this work has been presented at the 2nd International Symposium on *In situ* and On-Site Bioreclamation, San Diego, USA, April 1993).

B. MATERIALS AND METHODS

1. Organisms and Culture Conditions

The methanotrophs studied here (Table 1) were grown routinely at 25°C in a liquid nitrate-mineral salts medium (NMS) slightly modified from Cornish et al. (1984): 2 mM $NaNO_3$, 2 mM phosphate buffer (pH 6.8), 10 mM $NaHCO_3$, 150 M $MgSO_4.7H_2O$, 50 M $FeCl_3.6H_2O$, 50 M $CaCl_2.2H_2O$, 2 M $MnSO_4.4H_2O$, 2 M $ZnSO_4.7H_2O$, 2 M H_3BO_3, 1 M K_2SO_4, 1 M KI, 0.65 M $CoCl_2.H_2O$, and 0.4 M $Na_2MoO_4.2H_2O$. The medium was prepared with doubly deionized water (resistivity >18 MOhm cm^{-1}) and was essentially copper-free. The pH was adjusted to 6.8 with HCl. All glassware was acid-washed to avoid copper contamination. Cultivation was routinely performed under an atmosphere of 1:4 methane:air unless otherwise specified. Cultures were aerated by shaking at 200 rpm. The culture density was determined spectrophotometrically at 600 nm. Whole-cell protein content was determined by the microbiuret procedure of Munkres and Richard (1965).

2. Determination of sMMO-specific Activity

A modification of the naphthalene oxidation assay of Brusseau et al. (1990) was used to quantify sMMO-specific activity. Cell suspensions were diluted to an absorbance of 0.2 (at 600 nm). The diluted cultures were transferred in 1 mL aliquots to 10 mL screw cap test tubes, followed by the addition of 1 mL of saturated naphthalene solution (234 M at 25°C) (Verschueren 1983). The reaction mixtures were then incubated at 200 rpm on a rotary shaker at 25°C for 1 h. Controls included heat-killed cell suspensions and methanotroph cultures grown in the presence of 1 M $CuSO_4.6H_2O$. Triplicate samples were prepared. After incubation, 100 Liters of freshly prepared 0.2 percent (w/v) tetraotized *o*-dianisidine solution was added to the reaction mixture. Samples were

TABLE 1. NAPHTHALENE AND TCE TRANSFORMATION KINETIC PARAMETERS OBTAINED USING VARIOUS METHANOTROPHS.

Strain	Naphthalene		TCE	
	V_{max} (nmol h^{-1} mg protein^{-1})	K_s (M)	V_{max} (nmol min^{-1} mg protein^{-1})	K_s (M)
Methylosinus trichosporium OB3b (= ATCC 35070)[a,b]	328 ± 21[c]	40 ± 3	255 ± 62	126 ± 8
Methylosinus trichosporium OB3b (PP358)	305 ± 26	37 ± 5	278 ± 56	138 ± 13
Methylosinus sporium 5 (=ATCC 35069)	840 ± 47	96 ± 11	454 ± 84	178 ± 24
Methylococcus capsulatus Bath (ATCC 33009)	47 ± 5	84 ± 6	12 ± 3	249 ± 30
Methylomonas methanica 68-1[a]	551 ± 27	70 ± 4	360 ± 75	225 ± 13
Methylosinus sp. 2CC[d]	383 ± 27	83 ± 5	187 ± 28	200 ± 54
Methylosinus sp. 4CA	249 ± 11	23 ± 2	224 ± 46	89 ± 6
Methylosinus sp. 4CB	217 ± 3	24 ± 5	205 ± 35	96 ± 9
Methylosinus sp. 5CC	521 ± 48	38 ± 4	385 ± 72	120 ± 15
Methylosinus sp. 5CD	678 ± 14	32 ± 4	440 ± 48	136 ± 16
Methylosinus sp. 6CA	674 ± 20	24 ± 4	451 ± 77	99 ± 9
Methylosinus sp. 7CA	280 ± 14	59 ± 4	180 ± 61	153 ± 20
Methylosinus sp. 7CB	546 ± 26	48 ± 4	364 ± 68	117 ± 12
Methylosinus sp. 9BA	231 ± 20	54 ± 9	167 ± 20	177 ± 27
Methylosinus sp. 9CA	53 ± 3	69 ± 3	16 ± 5	310 ± 26
Methylocystis sp. 9BB	77 ± 13	65 ± 12	23 ± 6	246 ± 31
Unidentified group II methanotrophs:				
1C30A	72 ± 3	56 ± 3	29 ± 5	266 ± 22
1C30P2	405 ± 5	88 ± 2	239 ± 20	221 ± 40
1C30L	36 ± 4	72 ± 6	N.D.[b]	N.D.
1C50L1	405 ± 45	53 ± 5	268 ± 49	168 ± 17
2C10P	177 ± 14	89 ± 5	117 ± 37	188 ± 22
3C10P	420 ± 32	82 ± 5	280 ± 30	204 ± 17
3C50	578 ± 38	82 ± 4	347 ± 30	196 ± 23

[a]Data from Koh et al. (1993).
[b]ATCC, American Type Culture Collection, Rockville, Maryland, USA; N.D., no degradation detected.
[c]Parameters were calculated from triplicate analyses.
[d]Methanotrophs were isolated from either groundwater or sediment samples from a TCE- and tetrachloroethene-contaminated aquifer at the U.S. Department of Energy Savannah River Laboratories, South Carolina, USA (Bowman et al., 1993).

then immediately monitored spectrophotometrically at 525 nm. The intensity of diazo-dye formation is proportional to the naphthol concentration formed by the oxidation of naphthalene by sMMO. Wackett and Gibson (1983) determined the extinction coefficient of the naphthol diazo dye to be 38000 M^{-1} cm^{-1}. Naphthalene oxidation kinetics were determined by incubating cultures of the various methanotrophs listed in Table 1 with various levels of naphthalene ranging from 7.3 to 195 M for 1 hour and determining naphthalene oxidation specific rate (nmol h^{-1} mg $protein^{-1}$) at each concentration. Kinetic parameters were then determined by fitting the data to rectangular hyperbolic curves ($V = V_{max}S/(K_m + S)$ using the computer program DeltaGraph (DeltaPoint, Monterey, CA). Parameters were found to be in good agreement to values estimated from Lineweaver-Burk plots.

3. TCE Degradation Analyses

Cell suspensions were transferred in 1 mL aliquots into screw cap septum vials (14 mL; Pierce, Rockford IL) which were then closed with caps and Teflon®-lined silicone seals. TCE degradation was initiated by the addition of a saturated TCE aqueous solution (1100 mg/L or 8.36 mM at 25°C) (Verschueren, 1983). The vials were then inverted and incubated at 25°C on a rotary shaker at 200 rpm. After incubation (5-15 minutes) the reaction was terminated by the addition of 2 mL *n*-hexane containing 1 mg/L 1,2-dibromoethane as an internal standard. The undegraded TCE was extracted into the solvent phase by shaking and centrifugation (2000 grams, 20 minutes). TCE quantification was performed using a Shimadzu GC 9A gas chromatograph (Shimadzu Analytical instruments Co., Kyoto, Japan) equipped with a 1:1 split injector port operated at 220°C, a 30 m x 0.53 mm i.d. R_{TX} volatiles capillary column (Restek Corp., Bellefonte, PA) operated isothermically at 120°C with an electron capture detector at 220°C. Nitrogen was used as the carrier gas (flow rate 10 mL/min). The peak areas were integrated with a Shimadzu C-R6A Chromatopac. For determination of TCE degradation kinetics for the various methanotrophs listed in Table 1, the procedure of Oldenhuis et al. (1991) was followed.

4. Other Analytical Procedures

Concentrations of methane in cultures were determined by analyzing head space samples containing methane by gas chromatography using a Shimadzu GC 9 AM chromatogram equipped with a flame ionization detector and a 15m x 0.53 mm i.d. AT-1 capillary column (Alltech, Deerfield, IL) maintained at 60°C using nitrogen as the carrier gas (1 mL/min). Nitrate, copper, and iron concentrations were monitored following EPA standard methods (Franson, 1992). Phosphate was analyzed using a inorganic phosphorus kit supplied by Sigma Chemicals Co. (St Louis, MO).

5. Maximization Studies in NMS Media

a. Methane and oxygen availability

Methylosinus trichosporium OB3b was cultivated in 10 mL of NMS medium in 60 mL serum vials (Wheaton Inc., Millville, NJ) in which different initial dissolved methane concentrations were created by adding various amounts of methane with a syringe. The resultant dissolved methane concentrations ranged from 1.6 to 16.5 mg/L as determined by Henry's Law (Atkins 1986). In some experiments, nitrogen gas was added to the vials to vary the availability of dissolved oxygen (0.4-8.3 mg/L) in the presence of 20 percent methane. Specific growth rates were determined under these different conditions. sMMO specific activity was determined by the naphthalene oxidation assay.

b. Effect of nutrients

Various constituents in the NMS medium including nitrate, phosphate, $MgSO_4.7H_2O$, $FeCl_3.6H_2O$, $CaCl_2.2H_2O$, $MnSO_4.4H_2O$, $ZnSO_4.7H_2O$,$CoCl_2.H_2O$, and $Na_2MoO_4.2H_2O$ were either omitted or added at different concentrations, ranging from 0.01 up to 10 times the original concentration in the regular NMS medium. The effects on *Methylosinus trichosporium* OB3b specific growth rate and sMMO specific activity were assessed.

c. Effect of exogenous growth factors and carbon substrates

A series of experiments was undertaken to determine if carbon substrates or growth factors had a stimulatory effect on sMMO activity in *Methylosinus trichosporium* OB3b and in five other methanotrophs (Table 2). The carbon substrates were added to the NMS medium to obtain concentrations of 1 or 10 mM. The substrates tested included tricarboxylic acid intermediates and related compounds, including: acetate, DL-lactate, pyruvate, citrate, 2-oxoglutarate, succinate, malate, and, fumarate; serine pathway intermediates: L-serine, glycine, -hydroxypyruvate, and glyoxylate; D-glucose, various amino acids, and vitamins were also tested. The vitamin solution, a modification of Wolfe's vitamin solution (Balch and Wolfe 1976) consisted of: calcium pantothenate, niacinamide, thiamine-HCl, and riboflavin, all at 5 mg/L; d-biotin, folate, *p*-aminobenzoate, pyridoxal, and L-ascorbate all at 2 mg/L; pyridoxamine and pyridoxine, at 1 mg/L; and vitamin B_{12} at 0.1 mg/L.

d. Maintenance of sMMO activity

Previous experiments provided information on some of the requirements for obtaining high rates of sMMO activity in *Methylosinus trichosporium* OB3b and other methanotrophs. By utilizing this information, a series of experiments was designed to determine to what extent sMMO can be maintained in long term batch cultures of *Methylosinus trichosporium* OB3b. Maintenance experiments were performed in a simplified nitrate medium consisting of 2 mM $NaNO_3$, 2 mM phosphate buffer (pH 6.8), 50 M $FeCl_3.6H_2O$, and 50 M $MgSO_4.7H_2O$. The stability of sMMO activity in *Methylosinus trichosporium* OB3b was observed in long-term batch culture experiments. *Methylosinus trichosporium* OB3b was grown in 2 Liter flasks in 500 mL of NMS media. A set of flasks received methane (initial dissolved methane concentration was equal to 2 mg/L) at Time 0 and on each subsequent day of the experiment. The flasks were also sparged with filter-sterilized air periodically to prevent oxygen limitation. Another set of flasks was treated similarly except that methane was added only at Time 0 with no further additions for a period of 20 days. After 20 days addition of methane recommenced, with regular air sparging. A 20-mL sample was taken daily from both sets of flasks and analyzed for optical density, sMMO activity, nitrate, phosphate, and iron. Additionally, nitrate, phosphate, iron, and magnesium were added to all of the flasks to the original NMS medium concentration. The recovery and maintenance of sMMO specific activity was then monitored for up to 22 days.

TABLE 2. THE EFFECT OF VARIOUS VITAMINS ON sMMO ACTIVITY IN *METHYLOSINUS TRICHOSPORIUM* OB3b.

Vitamin	sMMO specific-activity (nmol h^{-1} mg protein $^{-1}$)
none added	317 ± 18
complete vitamin solution[a]	398 ± 20
pyridoxine	477 ± 25
d-biotin	472 ± 13
vitamin B12	451 ± 17
folate	348 ± 22
p-aminobenzoate	343 ± 19
pantothenate	339 ± 11
L-ascorbate	325 ± 16
pyridoxal	324 ± 21
pyridoxamine	313 ± 15
thiamine	236 ± 20
niacinamide	228 ± 10
riboflavin	227 ± 16

[a]The concentrations of the vitamins used were a 1:200 dilution of the concentrations in the complete vitamin stock solution (see text).

C. RESULTS AND DISCUSSION

1. Naphthalene and TCE Transformation by Methanotrophs

Strain selection and development for a TCE treatment system may be a useful start in an optimization process. As the development of $sMMO^+$ $pMMO^-$ copper tolerant mutants is feasible (Phelps et al., 1992) the problem of suppression of sMMO by copper (Tsien et al., 1989) can be avoided. Similarly the selection of strains exhibiting high rates of TCE transformation and a superior resilience to TCE toxicity is also possible. In this study significant variations were evident in the naphthalene and TCE transformation rates amongst several sMMO-producing methanotrophs examined (Table 1). A number of strains exhibited superior naphthalene or TCE transformation rates when compared to *Methylosinus trichosporium* OB3b and its $sMMO^+$ $pMMO^-$ constitutive mutant PP358 (Phelps et al., 1992). The maximal transformation rates (V_{max}) of naphthalene and TCE by the methanotrophs examined correlated in a linear fashion ($r^2 = 0.91$; $y = 36x + 22$). Overall, the TCE transformation V_{max} was approximately 36 times greater than the corresponding naphthalene oxidation V_{max}. This correlation appears to validate the naphthalene oxidation assay as a way of accurately quantifying sMMO activity. The assay can give an indication of the TCE transformation rate for an sMMO-producing methanotrophs at any given time. The naphthalene oxidation procedure has several advantages in that it does not require gas chromatography, is rapid, and convenient.

2. Effect of Methane and Oxygen Supply on sMMO Specific Activity

The specific-sMMO activity and TCE degradation rate in *Methylosinus trichosporium* OB3b was found to increase with increased dissolved methane concentrations. The greatest rates were achieved in the early stationary growth phase after 3 days incubation (Figure 1). Protein content in OB3b cells at this growth stage was found to be 0.70 ± 0.10 mg/mg cells. The initial rate of sMMO activity increase was about the same for the different methane concentration levels. The maximum levels of naphthalene oxidation were obtained with 16.5 mg/L methane at 308 ± 18 nmol h^{-1} mg $protein^{-1}$. After 4 days, enzyme activity started to decline with the decline dependent on the initial methane level. By 7 days enzyme activity was virtually absent in cultures grown with 1.6 mg/L or 3.3 mg/L methane with no residual methane detectable. However significant levels of sMMO activity were still present, as well as residual methane (0.5-1.7 mg/L), in the cultures initially supplied with 6.6 mg/L and 16.5 mg/L (Figure 1). To determine if the increase in sMMO activity was due to an increased availability of NADH supply, 20 mM sodium formate was included in the sMMO activity assays. It was found that sMMO activities of all the cultures were not appreciably stimulated by the formate until stationary phase was attained and methane became limiting. At this point the cultures with methane initially at 1.6 - 6.6 mg/L were stimulated to a level comparable to the 16.5 mg/L methane cultures (Figure 1). Thus increased sMMO-specific activity appears to be primarily promoted by the increased availability of reductant supply, i.e. NADH. This is probably due to increased dissimilatory methane oxidation (Rokem and Goldberg 1991). Studies with both *Methylosinus trichosporium* OB3b purified sMMO extracts (Dalton 1992) and whole cells (Alvarez-Cohen and McCarty 1991c) have found that enzyme activity declines rapidly if a hydroxylatable substrate, such as methane, is absent. The reduction in activity is thought to be related to a cellular NADH conservation mechanism mediated by the sMMO B protein preventing complete exhaustion of NADH pools by methane oxidation (Dalton 1992). The degree of reduction in sMM0-activity also seems to be affected by oxygen. Alvarez-Cohen and McCarty (1991c) noticed that low levels of oxygen had a stabilizing effect on sMMO activity when no substrate was present.

In our study oxygen did not seem to be a critical factor for high sMMO expression (Figure 2); instead oxygen had more bearing on cell growth rates and yields, a fact observed earlier by Park et al. (1991). A modest but significant increase in sMMO-specific activity did occur with ascending dissolved O_2 levels peaking at about 5 mg/L, while at higher O_2 levels, a slight decrease in specific sMMO activity was observable. Maintaining high concentrations of dissolved methane (>7 mg/L) could be implemented in two-stage dispersed bioreactors. The problems associated with methane reducing TCE-degradation rates due to competition (Broholm et al. 1992) can be avoided if TCE degradation takes place in the absence of methane. Oxygen concentrations ideally should be maintained at a level sufficient for high growth rates and sMMO-specific activity. Our study suggests 2 - 5 mg/L (dissolved O_2) would be sufficient.

3. Effect of Nutrients on sMMO Activity

The most critical media components for sMMO activity were nitrate, phosphate, and to a lesser extent iron, and magnesium. When completely deprived of a nitrogen source and thus actively fixing nitrogen, *Methylosinus trichosporium* OB3b still maintained high growth yields though it grew at a significantly lower growth rate (0.02 h^{-1}); however, sMMO activities were only 10 percent of the control cultures (data not shown). When fixing nitrogen, a significant proportion of the available NADH is apparently siphoned to the nitrogenase. Significant increases in sMMO activity were observed when 20 mM formate was added to nitrogen-starved cultures. With 0-2 mM nitrate present

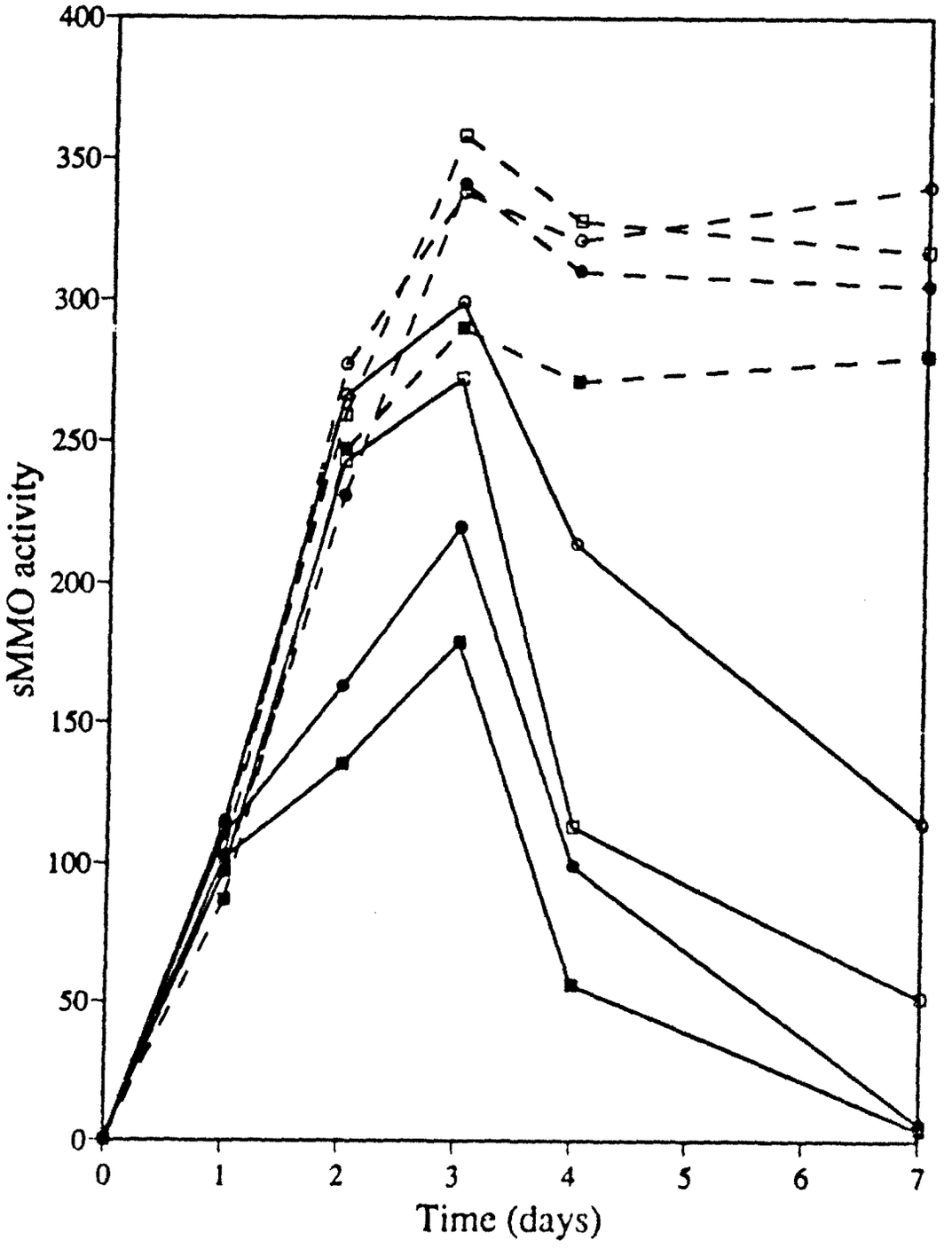

Figure 1. The effects of dissolved methane concentrations on sMMO specific activity in *Methylosinus trichosporium* OB3b. Specific sMMO activities were determined with resting cells with (----) and without (—) 20 mM sodium formate. The initial dissolved methane concentrations were 1.6 (■), 3.3 (+), 6.6 (□), and 16.5 (○) mg/l.

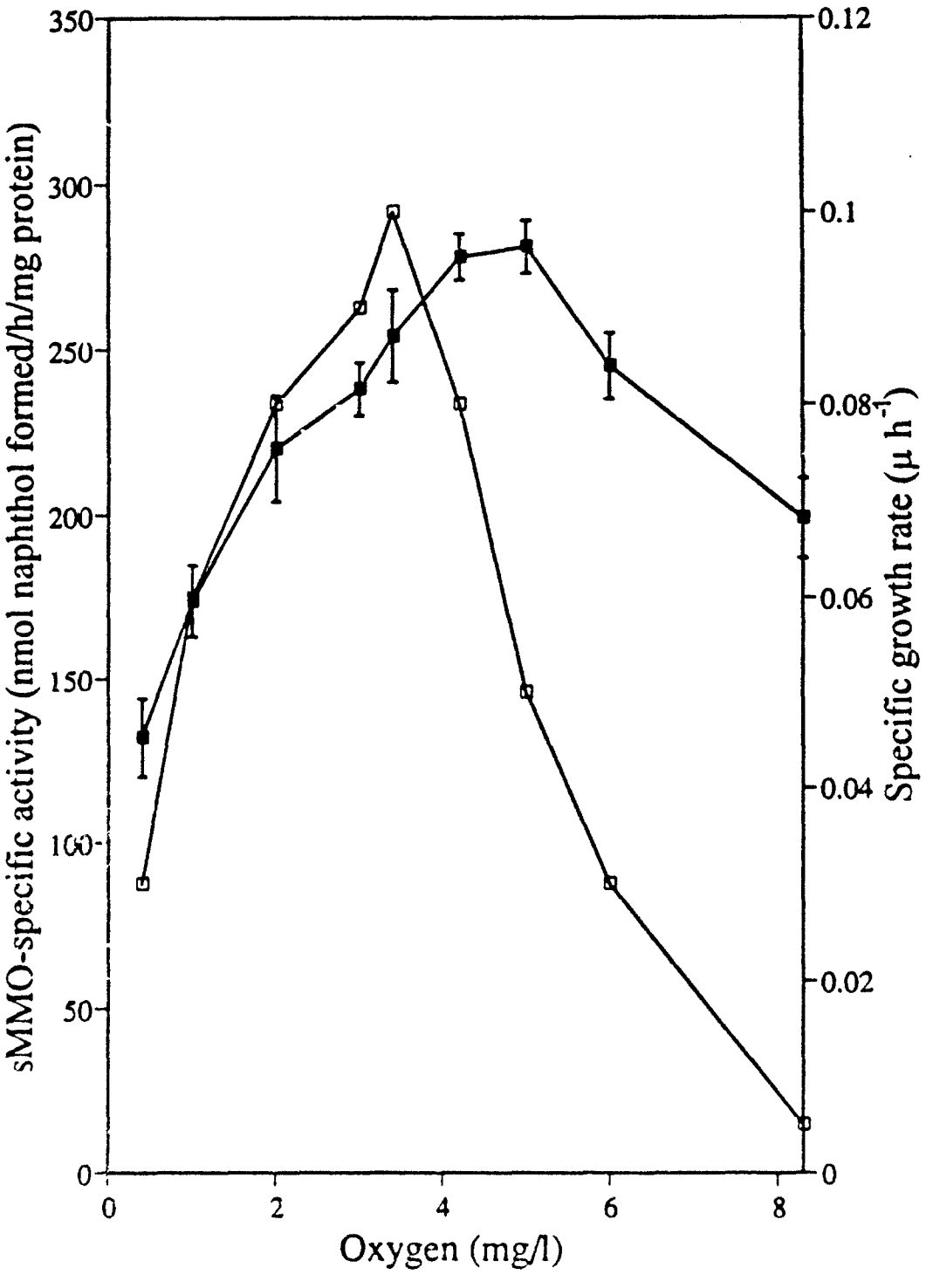

Figure 2. The effects of oxygen availability on specific growth rate (□) and sMMO specific activity (■) in *Methylosinus trichosporium* OB3b.

sMMO activity steadily increased (Figure 3). Between nitrate concentrations of 2 and 100 mM there was no significant further increase in sMMO activity, growth rates or yields. Phosphate, added at 2 to 25 mM maintains both sMMO and biomass levels at a similar level (Figure 3). Growing *Methylosinus trichosporium* OB3b at phosphate concentrations either lower or higher resulted in a significant decline in growth rate and sMMO activity (Figure 3). High concentrations of phosphate are known to inhibit methanol dehydrogenase activity (Mehta et al. 1987) leading to insufficient formaldehyde production for cell carbon assimilation or for dissimilation to generate NADH.

When iron was provided at less than 10 /μM there was a slight reduction in sMMO activity but a more significant decline in growth rate. This result has been previously reported by Park et al. (1991). However in this study it was possible to obtain stable and high sMMO activity at only 30 M $FeCl_3.6H_2O$. 50 M $MgSO_4.7H_2O$ seemed adequate to maintain maximal sMMO activity. When magnesium was not supplied, a moderate decrease in both growth rate and sMMO activity was observed (data not shown). Other trace elements, if absent from the growth medium, did not seem to affect sMMO activity or the growth of *Methylosinus trichosporium* OB3b and presumably are not vital for growth or sMMO synthesis. It was thus possible to define a simplified NMS medium to obtain high sMMO activity. The medium consisted of 2 mM $NaNO_3$, 2 mM phosphate buffer (pH 6.8), 50 M $FeCl_3.6H_2O$, and 50 M $MgSO_4.7H_2O$. Reasonable growth rates (0.08 h^{-1}) and yields (0.4-0.5 g cells/g methane) were obtained for OB3b in this medium. Ostensibly contaminated groundwater entering an sMMO-based "pump-and-treat" system could be supplemented with nitrate, phosphate and, if they are present in the groundwater only at low concentrations, iron and magnesium.

4. Effect of Supplementary Substrates

This study revealed that modified Wolfe's vitamin solution stimulated sMMO activity in *Methylosinus trichosporium* OB3b (Table 2). The addition of a 1:200 dilution of the Wolfe's vitamin solution gave the greatest increase in sMMO activity compared to unsupplemented controls. The addition of individual vitamins to the medium was performed to find the compounds responsible for the stimulation of sMMO activity. The highest levels of stimulation of sMMO activity occurred when vitamin B_{12} (0.5 /μg/L), d-biotin (10 /μg/L), or pyridoxine (10 /μg/L) were added (Table 2). Only a slight further increase in sMMO-specific activity occurred when these three vitamins were added together. Vitamins seemed to have an oligodynamic effect on sMMO activity. Excessive vitamin concentrations led to a degree of sMMO suppression. This was probably due to the influence of riboflavine, thiamine, and niacinamide which were inhibitory to sMMO activity (Table 2) and to growth rates. Subsequent experiments showed that vitamins do not act as artificial electron donors as is the case for formate and several other compounds (Leak and Dalton, 1983). The stimulation of sMMO only occurred when the cultures were actively growing in the presence of vitamin B_{12}, d-biotin, or pyridoxine. No significant increase in protein synthesis, growth rate, or growth yield was observed in the vitamin-supplemented cultures. Hypothetically, these compounds could be stimulating the activity of ancillary enzymes which may be indirectly associated with sMMO; alternatively, they may broaden the availability of NADH in the cell resulting in the increased sMMO-specific activity.

5. Maintenance of sMMO Activity

Cultures of *Methylosinus trichosporium* OB3b were grown in the simplified NMS medium (see above). The set of cultures that were provided methane only at the time of inoculation experienced methane limitation and a subsequent rapid decline in sMMO activity. No residual methane was

detected in these flasks after 13 days incubation. Those cultures given a relatively constant methane supply showed a lower rate of loss of sMMO activity (Figure 4). This decline slowed and eventually plateaued at a level equal to 45-50 percent of the original peak sMMO activity level (Figure 4). When methane limitation was removed in the methane-starved cultures (after 20 days incubation), sMMO activity was eventually restored to levels comparable to cultures which were continually maintained under methane (Figure 4). Samples taken from the cultures during the incubation were also tested with the addition of 20 mM sodium formate. No significant stimulation of sMMO activity was found in the cultures maintained with methane, while in those under methane limitation stimulation of sMMO activity was observed. The stimulated sMMO activity was approximately equal to those found for the cultures maintained with excess methane. Since sMMO can be maintained at a reasonably high activity in long-term cultures, bioreactor systems can be adapted to have a higher degree of cell recycling thus reducing the need to continually regrow cells. This approach could be used to improve the economic aspect of such systems; however, TCE toxicity also has to be considered.

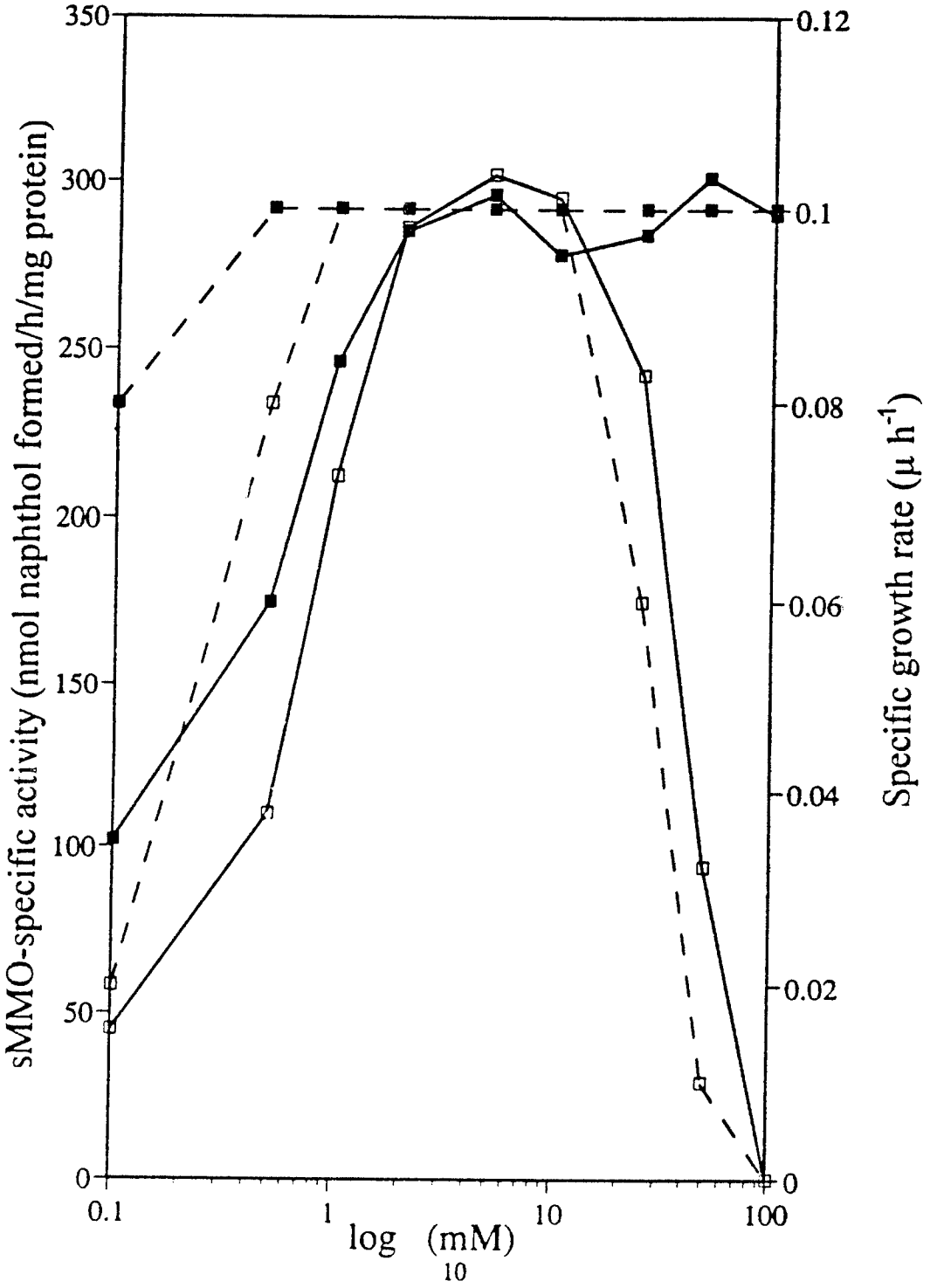

Figure 3. The effects of nitrate (■) and phosphate (□) levels on sMMO-specific activity (——) and specific growth rates (---).

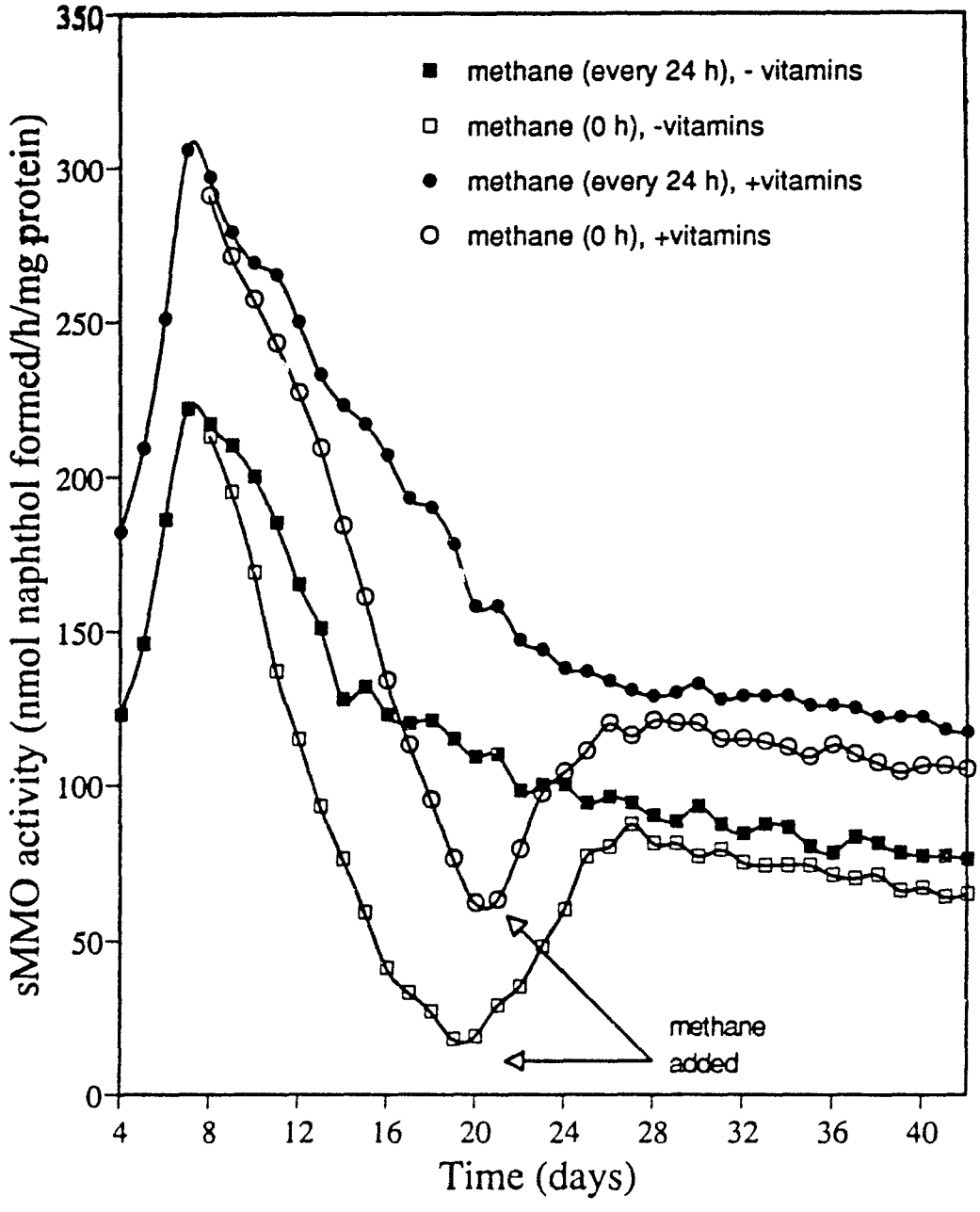

Figure 4. Maintenance of sMMO specific activity in *Methylosinus trichosporium* OB3b in NMS media supplied with 1:4 methane:air every 24 hours (■) and supplied with 1:4 methane:air only once at time 0 (□). Vitamin-amended (0.5 μg/l vitamin B_{12}, 10 μg/l d-biotin, and 10 μg/l pyridoxine) NMS media was also utilized with one set receiving methane every 24 hours (•) while another set received methane only once at time 0 (○). After 20 d of incubation the addition of 1:4 methane:air to the methane-starved cultures (□,○) recommenced.

SECTION III

RECOVERY OF sMMO AFTER TCE EXPOSURE

A. INTRODUCTION

The purpose of this work was to determine if formate could be used to increase either the rate of recovery of sMMO or the level of the enzyme after exposure of methanotrophic cultures to TCE in the absence of methane. Inhibition or destruction of sMMO during degradation of TCE by methanotrophs may be an element limiting TCE degradation. Two factors, lack of methane and damage to the MMO enzyme, can result in reduced MMO activity consequently, lowering TCE degradation capacity. A series of batch experiments was designed to determine the extent of sMMO inhibition, to determine if sMMO activity could be recovered and if recovery was enhanced by the addition of formate. After completion of these experiments (see below), it was evident that sMMO activity could be recovered following exposure to TCE. To apply these results to the bioreactor, design criteria had to be defined and batch experiments run in a modified form to simulate bioreactor conditions. In addition, the effects of several other chemicals anticipated to be effective in protecting sMMO were tested.

Activity of sMMO can be reduced by removal of the cultures from exposure to methane or by exposure to TCE. Although necessary to stimulate MMO production, the presence of methane can inhibit TCE degradation (e.g., Hanson *et al.*, 1989; Palumbo *et al.*, 1991) via competitive inhibition. It was expected that sMMO activity will decrease in the absence of the methane and the presence of the TCE. However, it should recover when removed from TCE and returned to the presence of methane.

The effect of formate on TCE degradation has been examined in many studies and often has a beneficial effect (Oldenhuis *et al.*, 1989; Eng *et al.*, 1991; Grbic-Galic *et al.*, 1991). In some studies, the positive effect has been attributed to provision of reducing equivalents believed to overcome rate limitations (Oldenhuis *et al.* 1989). The cells can utilize formate without MMO thereby supplying an energy source that does not compete with TCE.

During these experiments it became evident (see below) that, the presence of high levels of formate (>20 mM) during TCE degradation can protect the sMMO enzyme. The formate may function as a free radical scavenger. However, these high concentrations can inhibit sMMO recovery/synthesis. We examined what effect free radical scavengers had on TCE degradation. The chemicals tested were hypothesized to minimize the damaging effects of free radicals on the stability and recovery of the sMMO enzyme. However, we did not identify these compounds as free radical scavengers and refer to them as protectant chemicals.

B. METHODS

1. Cultures and Growth Conditions

To simulate the culture conditions likely to be present in a bioreactor, these experiments were run with a mixed culture of *Methylosinus trichosporium* strain OB3b, a Type II obligate methanotroph and a heterotroph. A modification (Little *et al.*, 1988) of NATE medium (Whittenbury *et al.*, 1970)

was used to grow the cultures. Further modifications in these experiments were substitution of additional nitrate for the ammonia, and elimination of copper from the trace metal formulation.

Cells for sMMO inhibition and recovery experiments were obtained from the mixed cultures maintained in an air lift bioreactor (Kontes) continuously flushed with a gas mixture containing 3 percent methane in air. Optical density (λ= 660 nm) (OD_{660}) and sMMO were measured prior to use of the cultures in these experiments because growth phase appeared to affect sMMO activity (unpublished data). It appeared that methane and oxygen were in excess because a plateau in optical density was reached; however, optical density could be increased further with the addition of supplementary inorganic nutrients (in the same proportions as in the original media) (unpublished data).

2. Analytical Procedures

Relative sMMO levels were determined by the naphthalene oxidation assay (Brusseau *et al.* 1990). The initial OD (OD_i) is used as a biomass indicator. The change in OD during the sMMO assay (ΔOD) is used as an indicator of the total sMMO activity and the change in OD divided by the initial OD ($\Delta OD/OD_i$) is used as an indicator of biomass specific activity. These units of optical density can be converted to moles of naphthol per hour per mg of cells using the following relationships. In the OD_i range of the batch experiments a linear relationship exists between OD cell concentration with an OD_i of 0.1 equivalent to approximately 110 mg cells/L (unpublished data). The relationship between ΔOD and naphthol concentration was also linear with an extinction coefficient of 38,000 mole/cm. Thus, dividing ΔOD by 38,000 and dividing again by the cell concentration (in our experiments usually about 100 mg/L) and the incubation time (usually 1 hour) gives the biomass specific naphthol production rate. Using these relationships an approximate factor of 18 can be used to convert $\Delta OD/OD_i$ to nmoles of naphthol/mg cells.

TCE was analyzed using a Sigma Model 2000 (Perkin Elmer, Norwalk, Connecticut) gas chromatograph (GC). The GC was equipped with a capillary column, an electron capture detector. The detector temperature was set at 200°C, and the oven temperature was set at 100°C. TCE had a retention time of 3.4 minutes and was measured in 30 µL samples of the headspace gas. Standards in triplicate consisted of NATE plus TCE added for a final concentration of 0.5, 1.0, and 5.0 mg/L. Autoclaved cells plus TCE (1.0 mg/L) were used to control for adsorption to the biomass.

3. Experimental Procedure

The inhibition and recovery experiments consisted of exposing OB3b cells containing high levels of sMMO to TCE in the absence of methane using 40 mL EPA vials with Teflon®-lined septa (Supelco, Bellefonte, PA) and a liquid volume of 5 mL. The cells were contained for 18 to 24 hours and the indicators of sMMO activity and TCE degradation were measured. Following exposure to TCE, cultures were removed from contact with any residual TCE and various treatments (described below) were applied to examine recovery of sMMO activity. TCE was added as an aqueous saturated solution at 5, 10, or 20 µl, yielding a nominal TCE concentration 0.5, 1.0, and 2 mg/L respectively (assuming that the TCE was in the liquid phase). Actual concentrations in the liquid were lower due to partitioning into the gas phase. Vials were incubated inverted on a shaker. Headspace measurements were used to quantify TCE concentrations during the exposure period and to document TCE degradation. After a 24- or 48- hour recovery period, indicators of sMMO activity was remeasured. During this time cultures were exposed to formate or formate plus methane. A

total of 10 mL of methane was added to the vials in the recovery experiments. Since the headspace was 35 mL, the methane concentration in the liquid phase approached saturation. A series of preliminary (scoping) experiments was performed to determine the appropriate concentrations of formate and TCE and exposure times for use in these recovery experiments.

4. Scoping Experiment

A total of 24 sets of triplicate vials was used in an experiment with all recovery in the presence of methane. The first part of this experiment was designed to quantify reduction in sMMO activity resulting from exposure to TCE and lack of methane. A group of three treatment sets and one set of controls, with three replicates per set, was used to examine the effect of bacterial culture dilution, TCE exposure, and lack of methane on sMMO activity before the recovery period.

Both diluted and undiluted samples were used in the main portion of this experiment that examined recovery after exposure to the TCE. The dilutions were made and compared to undiluted cultures to ensure that reproducible densities could be used in subsequent experiments. Seven treatments contained undiluted cultures and were used to examine the effect of TCE (0, 1 and 2 mg/L) on recovery of sMMO activity in the presence of 8 mM formate and in the presence of methane (Table 3). Of these seven sets, three sets were controls and were not exposed to TCE, methane starvation or formate. Two of these three controls were measured at 24 hours and one was set at 48 hours to verify the persistence of the sMMO during the recovery period. Four treatment sets (1 and 2 mg/L) were exposed to methane and formate during the recovery period (2 for 24 hours and 2 for 48 hours) after exposure to TCE. A second group of seven sets of cultures was diluted to 0.2 OD and was treated similarly to the first group, except that TCE levels were 0.5 and 1.0 mg/L and the formate concentration was 16 mM (Table 3). The final group consisted of four sets of vials with cultures diluted to an OD of 0.2 and 0.4 exposed to either 0.5 mg/L (two vials) or 1.0 mg/L (two vials) of TCE and recovery was measured at either 24 or 48 hours (Table 3). There were no controls for persistence of sMMO run at this dilution. TCE degradation was monitored by GC analysis during the exposure period. Two other sets were used for controls for loss of TCE. These sets contained only TCE (0.5, 1.0 and 2.0 mg/L) and media or TCE (2.0 mg/L) and autoclaved cells. Enzyme level comparisons among the groups were made to determine if dilution affected enzyme recovery.

5. Factorial Experiment and Related Treatments

A more thorough experiment was designed to build upon the results of the above scoping experiments. As in the previous experiments, appropriate controls without TCE and with methane were used to compare the sMMO activity after exposure to TCE (0.5, 1.0, and 2.0 mg/L) in the absence of methane. For the factorial sMMO recovery experiment all treatment sets (in triplicate) were set up with undiluted culture. As in the previous experiments, all recovery took place in the presence of methane (10 mL). These sets were set up as a three way (3 x 3 x 2) factorial analysis of variance including the effect of TCE concentration (0.5, 1.0 and 2.0 mg/L), formate concentration (0, 8, and 16 mM.), and the effect of recovery time (24 or 48 hours). An additional four sets of controls not exposed to TCE verified the persistence of sMMO activity with one or two methane additions over the 48-hour recovery period and the addition of 32 mM formate with the methane. As in the scoping experiments, two treatment sets were GC controls.

TABLE 3. NUMBER OF TRIPLICATE SETS CONTAINING 3 REPLICATES USED IN EACH TREATMENT OF THE SCOPING EXPERIMENT USING CULTURES THAT WERE UNDILUTED (UD), DILUTED TO AN OD OF 0.4, OR DILUTED TO AN OD OF 0.2 AT FORMATE LEVELS OF 0, 8.0, OR 16 MM AFTER EXPOSURE TO 0, 1.0, OR 2.0 mg/L TCE[1].

Dilution	TCE (mg/L)	Formate (mM)	Number of Sets at 24 h	Number of Sets at 48 h
UD	0	0	2	1
UD	1.0	8	1	1
UD	2.0	8	1	1
0.2	0	0	2	1
0.2	0.5	16	1	1
0.2	1.0	16	1	1
0.2	0.5	0	1	1
0.4	1.0	0	1	1

[1]An additional 4 sets (not shown on the table) were used to determine the effect of 24 h of TCE exposure on indicators of sMMO.

6. Statistical Analysis

Statistical analysis was done using version 6.0 of the SAS software (SAS Institute 1985) on a personal computer. The variables used in the analysis were OD_i, Δ OD, and Δ OD/OD_i. The GLM procedure of SAS was used for the analysis.

One way analysis of variance with Duncan's multiple range test was used to analyze data from the scoping experiments. In the scoping experiment the effect of dilution (undiluted, diluted to OD of 0.4, and 0.2) and the effect of formate (0, 8, 16 mM) were confounded so that no statistical determination could be made on the effect of formate on recovery independent of the dilution effect.

Two types of analyses were performed on the data from the factorial recovery experiments. Duncan's multiple range test was use for testing of differences in selected means. Comparisons of treatments (vials with TCE added) to controls at the start of the recovery period were made as part of a complete one way analysis of variance that treated each group of three replicate vials as an individual treatment. Comparisons among treatments, which were never exposed to TCE, at 24 hours and 48 hours were also made as part of this analysis.

Also, overall treatment effects were examined using part of the data in a 3-way (3 x 3 x 2) factorial analysis of variance including the effect of TCE level, formate concentration, and the effect of recovery time. Significance of individual effects was tested based on the Type III sum of squares

(to eliminate the effect on entry order on the analysis) and were termed significant if they were above the 95 percent confidence level. Two way interaction terms were included in the analysis. Due to experimental difficulties two of the 18 cells in the factorial analysis only had two replicates.

7. Simulation of Bioreactor Conditions

To relate the batch experiments to bioreactor operations, a second set of batch experiments was designed after the bioreactor was operational and operating conditions had been established. The generation of sMMO activity shown in batch experiments (see below) was likely not due to new cell growth as there was no increase in overall optical density, but was primarily due to new enzyme synthesis from the existing biomass. It is also possible that the enzyme was present but was inactivated by the formation of the TCE epoxide. Thus, enzyme recovery could also be due to a reconfiguration into the active state. Whatever the mode of action, the enzyme activity recovered within 24 hours and exceeded initial start levels.

The bioreactor has zero headspace, however, batch experiments were carried out in vials with a headspace (described in previous section). To compare batch experiments to bioreactor conditions, we assessed the effect of headspace volume (i.e., oxygen and methane concentrations) on enzyme stability/recovery. Cell suspensions of 5, 10 and 15 mL were added to 40-mL EPA vials. One set was inoculated with methane (as described above) and a second set without methane. The enzyme activity was measured after 1, 2, 4, 6, 24, 48 and 72 hours.

8. "Protectant" Chemicals/Free Radical Scavengers and sMMO Activity

The chemicals tested were citric acid (2 mM), ascorbic acid (2 mM), formic acid (2 mM) and calcium carbonate (trace). These compounds were added with the TCE and its degradation followed by GC analysis. Following TCE degradation, the enzyme was recovered by the addition of formate and methane addition. The system was assayed 12 hours later and enzyme levels were compared to controls.

C. RESULTS

1. Scoping Experiment

After a 24 hour exposure to TCE and dilution of biomass the indicators of sMMO activity and the sMMO per cell for all TCE treatments were significantly different from the 0 mg/L controls maintained on methane (Figure 5A). Based on the use of Duncan's multiple range test on the whole experiment, differences among the four means of greater than 0.0858 for ΔOD and 0.100 for $\Delta OD/OD_i$ are significantly different at the 95 percent confidence level. There were no significant differences among the treatments starved for methane and exposed to TCE (Figure 5A). Maximum sMMO activity observed in these experiments was approximately 7.6 nmoles napthol/mg cells/h.

After 24 hours, there was significant recovery of the sMMO activity indicators and biomass specific sMMO activity after methane and 8 mM formate addition in the undiluted samples exposed to 1.0 and 2.0 mg/L TCE (Figure 5B). At 24 and 48 hours (Figure 5C) the indicators of sMMO activity in all undiluted treatments were significantly higher (see above) than time zero treatments exposed to TCE. There was no significant difference in the recovery at 24 and 48 hours; however, additional methane was added only at the start of the recovery period.

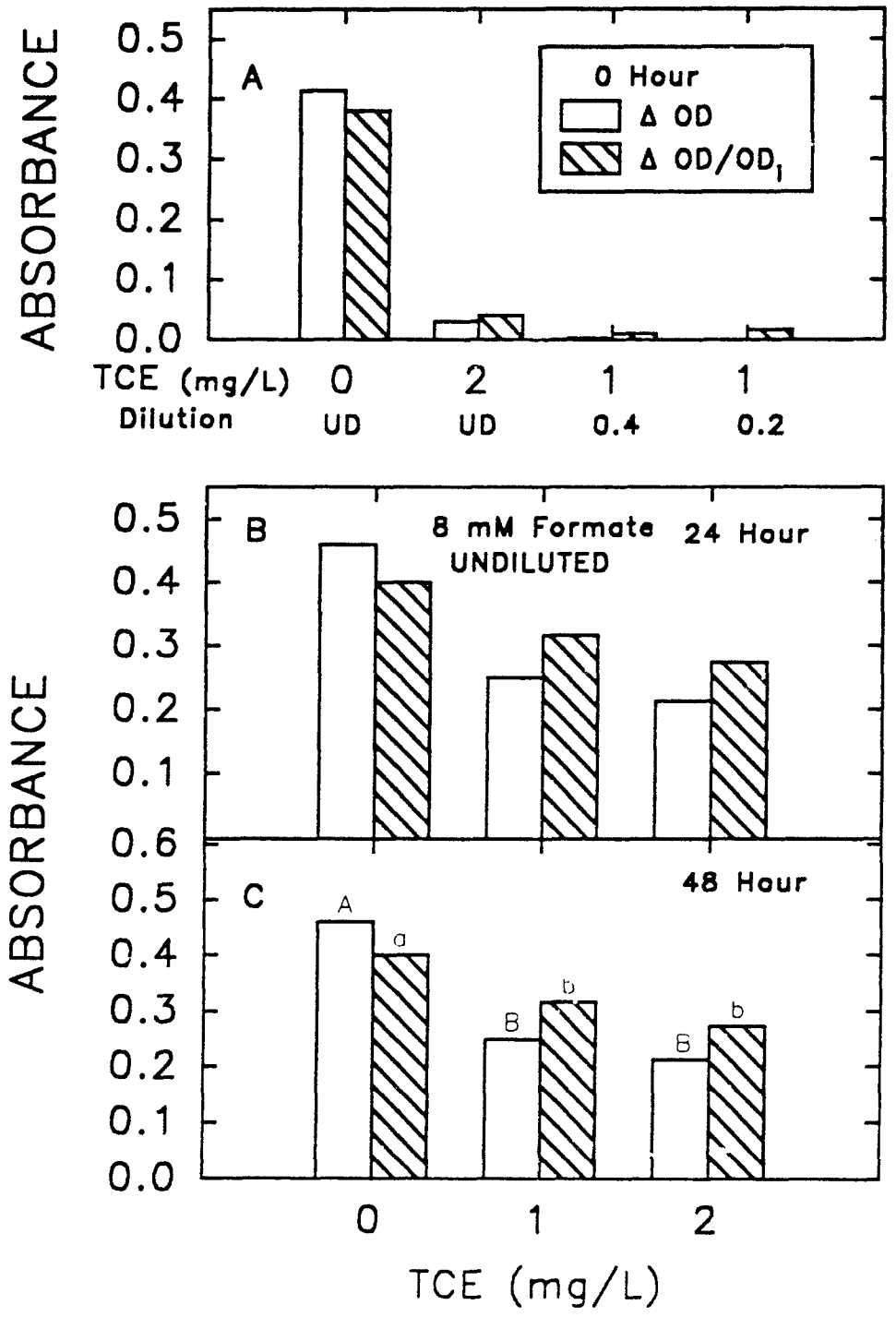

Figure 5. Effects of TCE at 1 and 2 mg/L and lack of methane on indicators of sMMO activity (Δ OD) and biomass specific sMMO activity (Δ OD/OD$_i$). A. Effect of 24 hour exposure to TCE on the sMMO activity indicators for cultures that were undiluted (UD), cultures that were diluted to 0.2 OD and cultures that were diluted to 0.4 OD. B. Effect of 24 hour recovery with exposure to 8 mM formate and methane for undiluted samples. C. Effect of 48 hour recovery with exposure to 8 mM formate and methane for undiluted samples.

Diluted samples also showed a significant increase in specific sMMO activity compared to the time 0 TCE exposed treatments (Figure 6) but biomass specific activity was lower than with the undiluted cells (Figure 5). Biomass specific activity only reached a maximum of approximately 1.8 nmoles napthol/mg cells/h in the 0.2 dilution (Figures 6A and 6B) and of approximately 6.3 nmoles napthol/mg cells/h in the 0.2 dilution (Figures 6A and 6B). When the greatest dilution (0.2 OD) was incubated with 16 mM formate and methane, the indicators of sMMO activity were close to zero after 24 hours of recovery which followed exposure to TCE for 24 hours (Figure 6A). This was the case for all treatments but sMMO activity was significantly higher in the treatment that had not been exposed to TCE. However, by 48 hours (Figure 6B) indicators of sMMO activity, particularly $\Delta OD/OD_i$ (at both levels of TCE addition) had risen substantially and enzyme activity was not significantly different between the two TCE levels. At the 0.4 OD dilution there was some activity evident by 24 hours (Figure 6C) and there was substantial activity by 48 hours (Figure 6D).

2. Factorial Experiment

Before the 24-hour recovery period but after TCE exposure and methane removal, the indicators of total sMMO activity and the biomass specific sMMO for the 0.5, 1.0, and 2.0 mg/L TCE exposure treatments were significantly lower than controls incubated with methane and without TCE (Figure 7A). Biomass specific sMMO activity in the controls was in the range of 4.5 nmoles napthol/mg cells/h. There were no significant differences among the three levels of TCE addition for indicators of sMMO activity or for biomass specific sMMO activity.

In treatments not exposed to TCE, but in the presence of methane 24 hours after the start of the recovery period the indicator of total sMMO activity had gone up significantly (Figure 7B) but biomass specific activity (OD/OD_i) had not. After 48 hours of recovery, a similar treatment that had received additional methane at 24 hours was significantly lower for both activity indicators than the initial or the 24 hour measurements. A similar treatment that did not receive a second methane injection at 24 hours showed virtually no sMMO activity 24 hours later (48 hours after the start of recovery). Also after 48-hour recovery, a treatment with 32 mM formate and methane addition at the start of the recovery was not significantly different in either activity measure than the treatment that received two methane additions over the same period. The highest biomass levels were seen 48 hours after initiation of the recovery period in the treatments not exposed to TCE. As expected, the treatment that received two injections of methane and in the treatment that received both methane and formate were not significantly different but were both significantly higher than all other treatments. The lowest biomass levels were measured at the initiation of the recovery period in the three treatments exposed to TCE.

The three-way analysis of variance indicated that formate level had a significant effect ($p >$ 95 percent) on recovery of the indicators of total sMMO activity and specific sMMO activity but not on biomass (Table 4). There is a significant interaction of formate with time in the analysis of the indicators of both the total sMMO activity and biomass specific sMMO. This interaction probably arises from the lack of an effect of formate on recovery at 48 hours (Figure 8A). Thus it appears that formate has a greater effect on the rate of recovery than on the final recovery level.

The analysis also indicated the significant effect TCE concentration on recovery. Effects of TCE conclusions on sMMO activities during the recovery period were most pronounced at zero or 0 mM formate and were least pronounced at 8 mM formate (Figure 8B). TCE had a significant effect on all three independent variables.

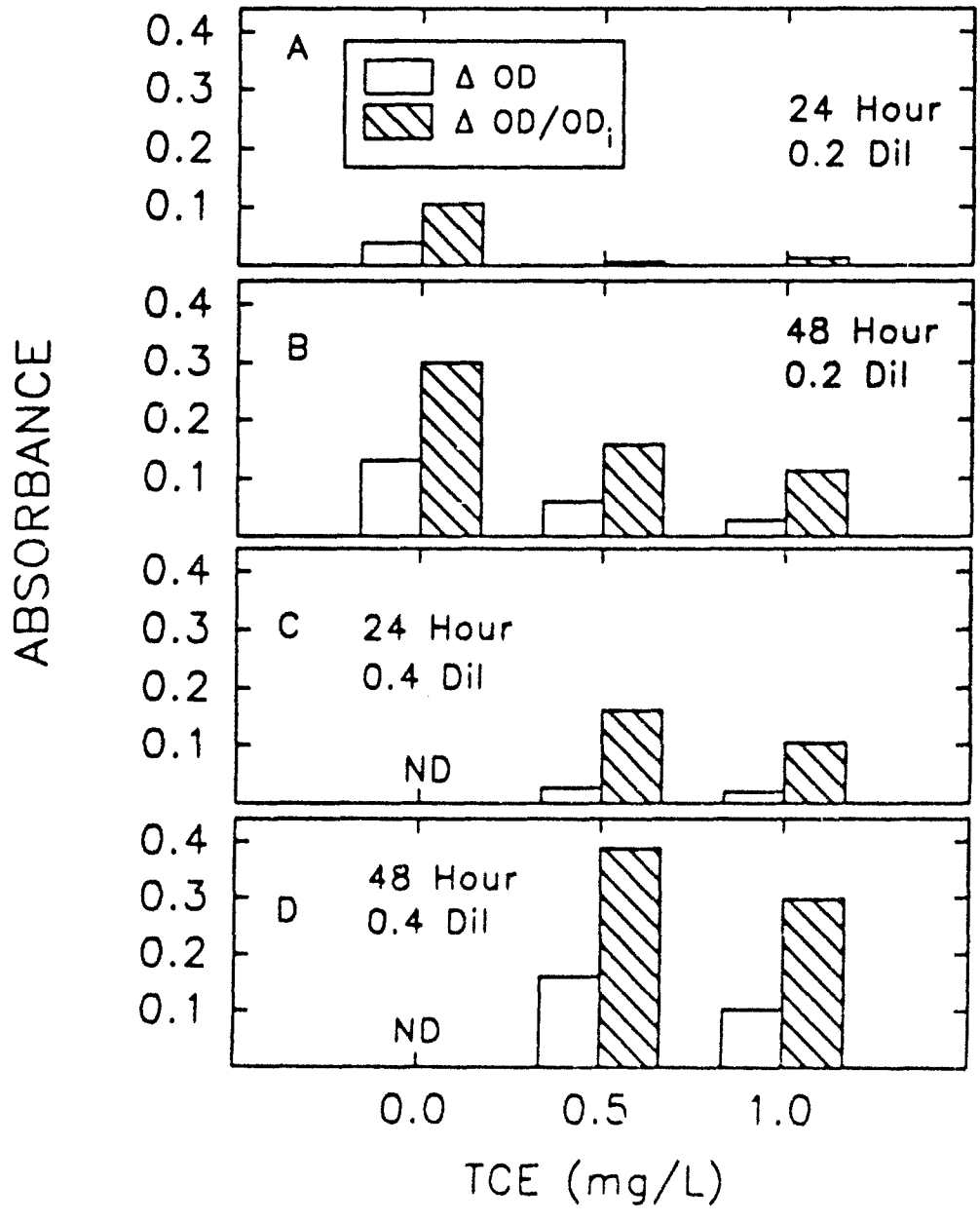

Figure 6. Indicators of sMMO activity (Δ OD) and biomass specific sMMO activity (Δ OD/OD$_i$) after 24 hours (A) and 48 hours (B) of recovery after exposure to TCE and lack of methane on cultures diluted to 0.2 OD and exposed to 16 mM formate and methane during recovery.

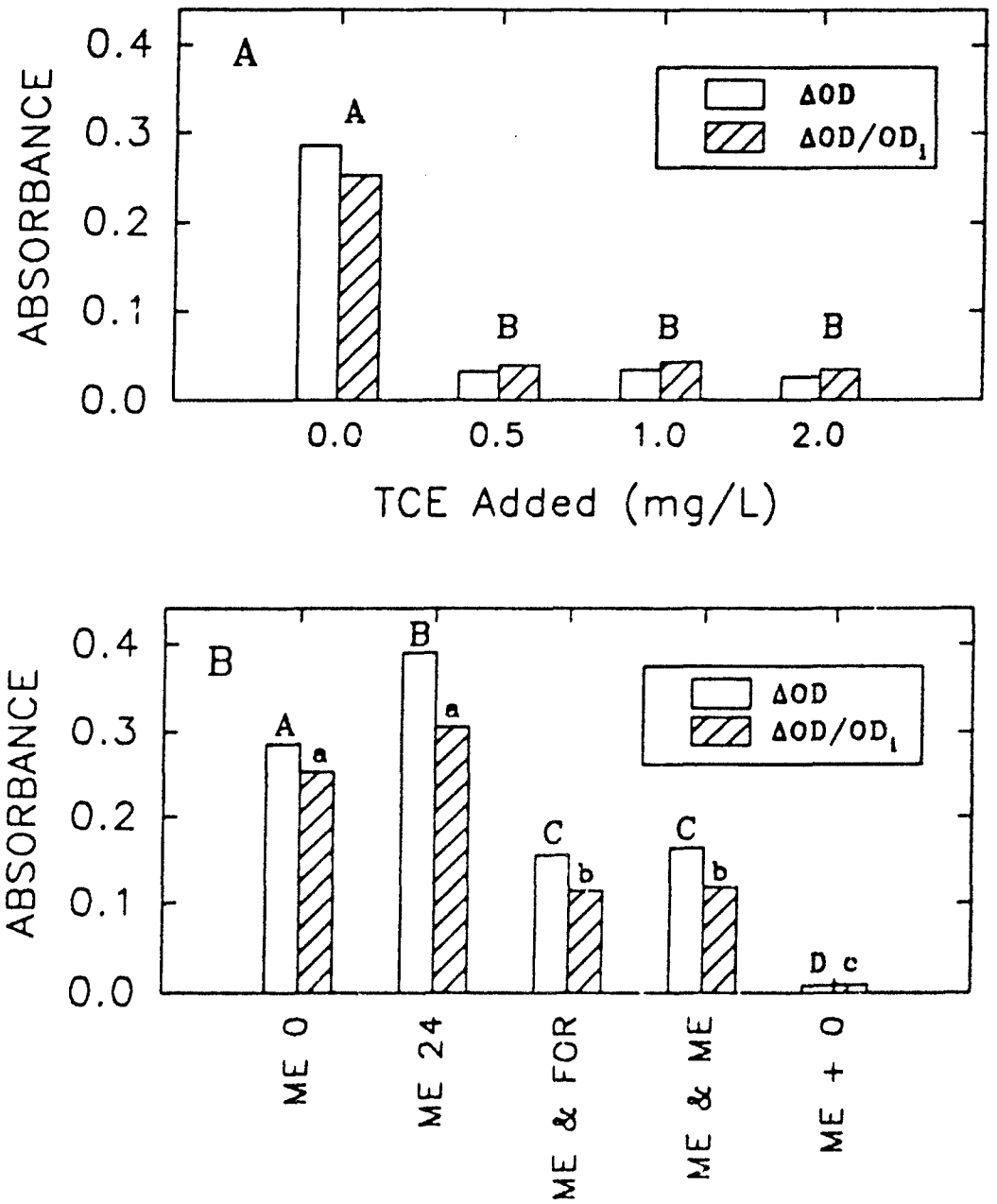

Figure 7. Effect of TCE, methane, and formate on indicators of sMMO activity (Δ OD) and biomass specific sMMO activity (Δ OD/OD$_i$). Capital letters designate differences in Δ OD or differences in both Δ OD and Δ OD/OD$_i$ if no lowercase letters are given to specifically designate differences in Δ OD/OD$_i$. A. Reduction in sMMO activity indicators after 24 hours exposure to TCE. B. sMMO activity in cells not treated with TCE at time 0 (ME 0) and at 24 hours (ME 24), and cells exposed to TCE and allowed to recover for 48 hours with exposure to methane and formate (ME + FOR). Methane added at 0 and 24 hours (ME + ME), and methane added at 0 hours only (ME + 0). Treatments with the same letter are not significantly different ($p < 95$ percent). Based on Duncan's multiple range test on the whole experiment, differences among the five means of greater than 0.005 for Δ OD and 0.083 for Δ OD/OD$_i$ are significantly different at the 95% level.

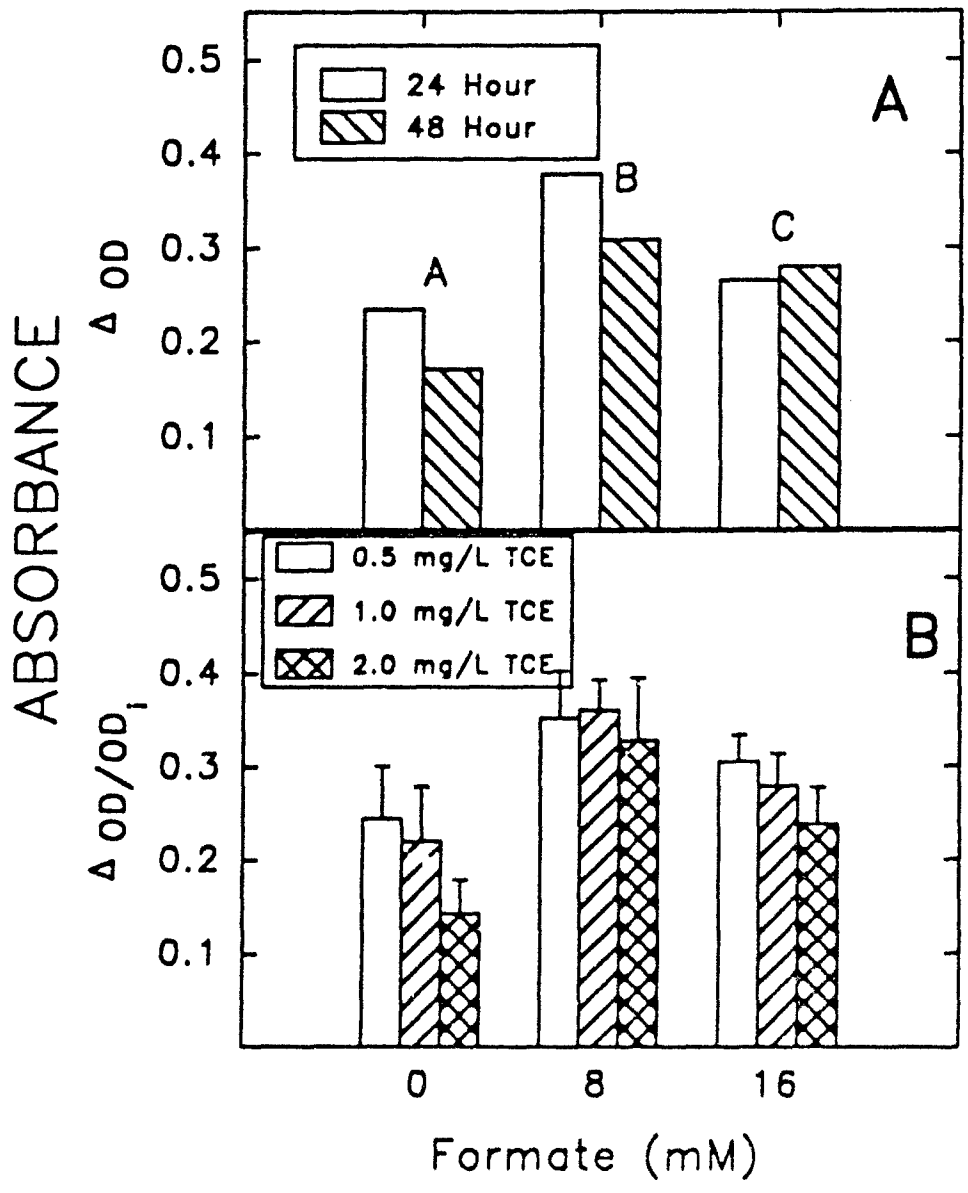

Figure 8. Effect of formate concentration, time, and TCE concentration on indicators of sMMO activity (Δ OD) and biomass specific sMMO activity (Δ OD/OD$_i$). A. Effect of time of recovery and formate concentration on Δ OD. Significant differences in formate treatments are indicated by differences in capital letters. There is no significant differences between the 24 and 48 hour recovery treatments. B. Effect of formate and TCE concentration on Δ OD/OD$_i$. Error bars indicate standard deviations.

The overall effect of time on the recovery process was limited to the indicators of biomass and biomass specific sMMO activity. Again, the significant interactions of formate with time for the indicators of sMMO activity and biomass specific sMMO activity showed that the effect of time was not consistent over the three formate levels (Figure 8).

3. Simulation of Bioreactor Operation

An important aspect of the bioreactor operating conditions was the lack of headspace. In the batch experiments, vials without methane had significantly decreased enzyme activities after 6 hours while those with methane maintained high levels of activity for 24 hours (Figure 9). Evidently over 48 hours there was a relationship between cell suspension volume and enzyme activity. Vials containing 15 mL of cells maintained their activity longer than those with 5 mL vials.

Recovery of the sMMO activity was accomplished in 8-12 hours (Figure 10). Cell suspensions were treated with 20 mg/L TCE and following degradation (confirmed with GC analysis) enzyme activity was recovered by the addition of methane and formate. Initially the enzyme activity decreased by half after TCE exposure and increased to control treatment levels after eight hours of recovery and almost double control levels after 12 hours of recovery (Figure 10).

The recovery of the sMMO enzyme activity appears to be a dose dependent phenomena. Previously we determined that 0.5-5 mg/L TCE could be degraded by OB3b and the enzyme activity was recoverable. Recent experiments used cell suspensions with very high enzyme activities that degraded 5 mg/L TCE within two hours. Therefore, we increased the TCE concentration to 10, 20, and 50 mg/L. The TCE was degraded to below detection levels for all but the 50 mg/L treatment.

TABLE 4. ANALYSIS OF VARIANCE FOR THE FACTORIAL EXPERIMENT ON INDICATORS OF SMMO ACTIVITY (Δ OD) AND BIOMASS SPECIFIC SMMO ACTIVITY (Δ OD/OD_i).

	Δ OD			Δ OD/OD_i		
Source	DF[1]	MS[2]	F[3]	DF	MS	F
TCE	2	0.0293	17.9**	2	0.0196	13.7**
Formate	2	0.0968	59.0**	2	0.0875	61.1**
Time	1	0.0021	1.3	1	0.0184	12.84**
TCE*Formate	4	0.0020	1.2	4	0.0024	1.68
TCE*Time	2	0.0003	1.9	2	0.0025	1.74
Formate*Time	2	0.0103	6.3**	2	0.0097	6.76**

[1]DF = degrees of freedom.
[2]MS = mean square.
[3]F= mean square of the model or effect divided by mean square of the error.
**Significant at the 99 percent level or better.

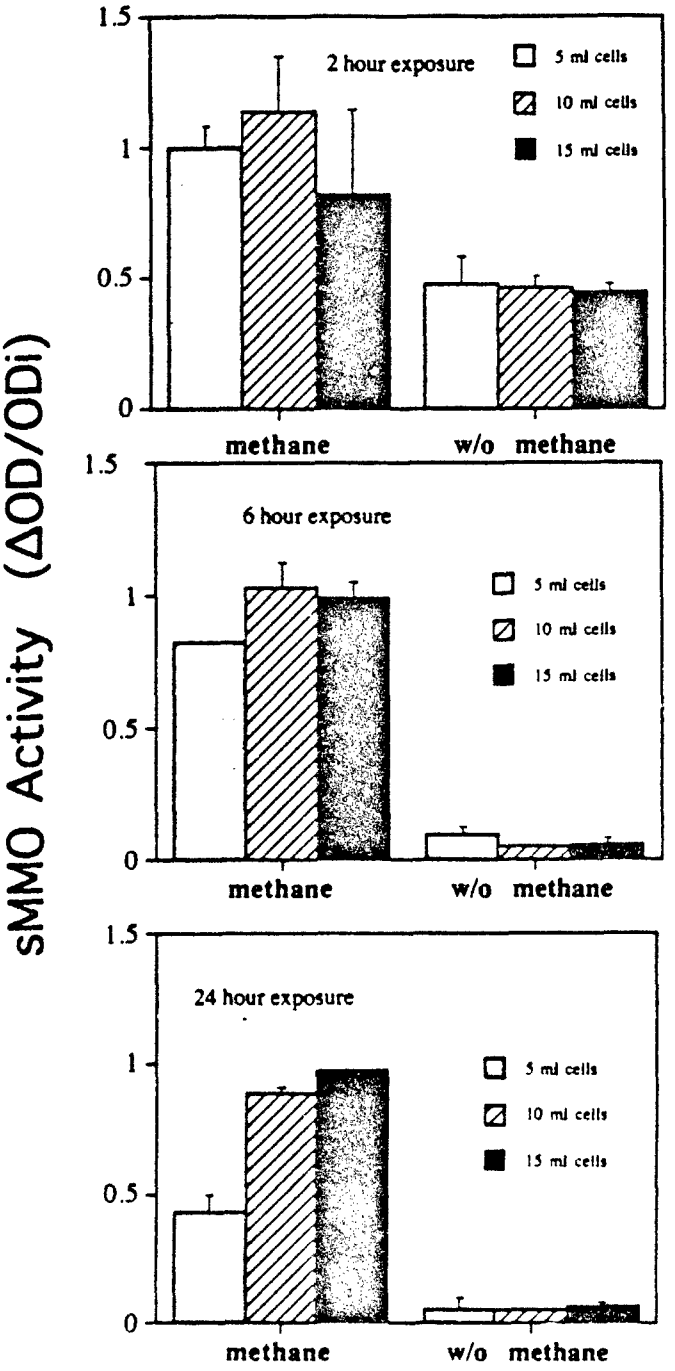

Figure 9. Effect of headspace on sMMO stability. Vials containing 5, 10 and 15 mL cell suspension were treated with and without methane. Enzyme levels assayed at 1, 6 and 24 hours (n=2). Enzyme activity reported as a change in OD/OD initial.

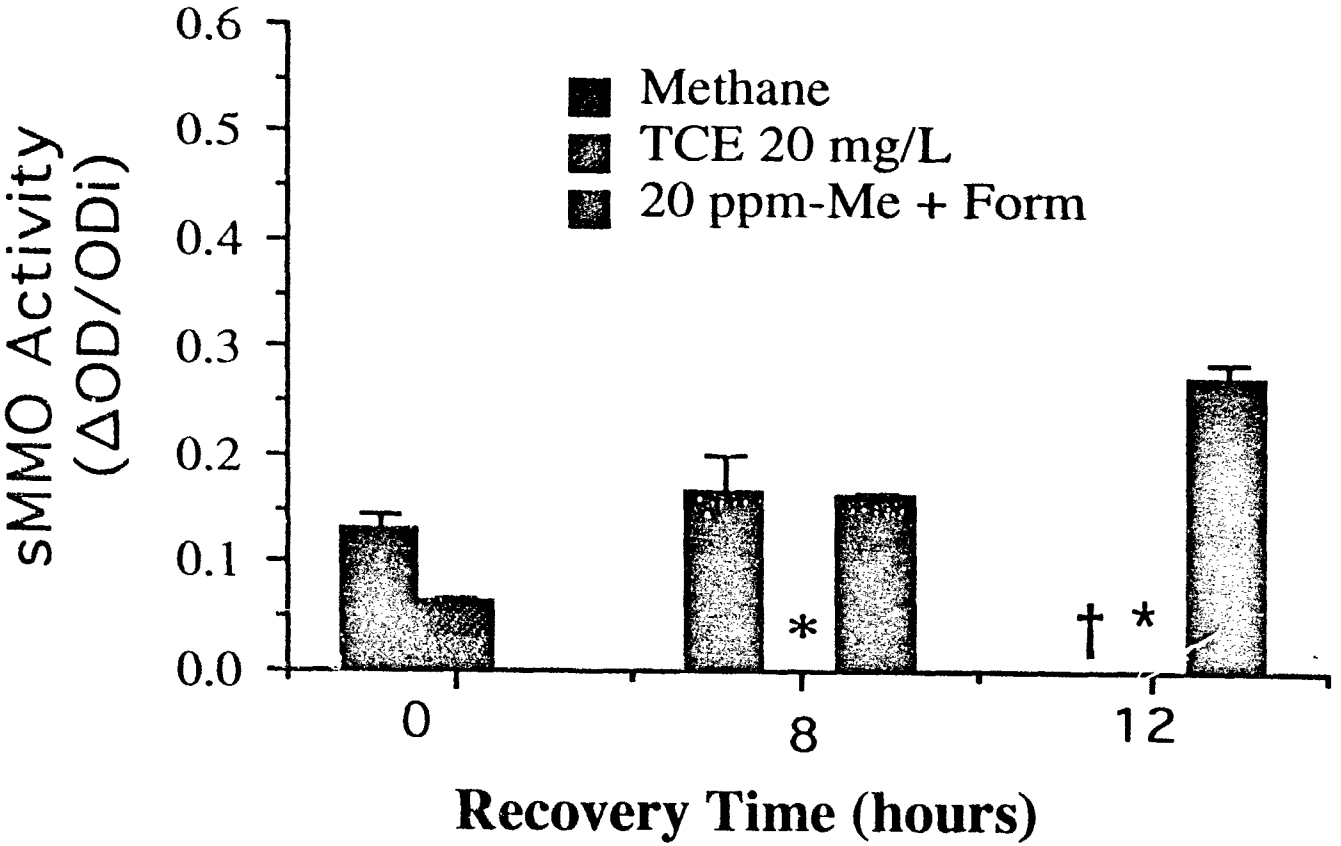

Figure 10. Enzyme activity assayed after exposure to 20 mg/L TCE and recovered with formate and methane (n=2). Enzyme activity reported as a change in OD/OD initial.

In the latter case, the remaining TCE was vented and the recovery regime of 4 mM formate plus methane was initiated. The amount of enzyme activity recovered was related to the initial TCE concentration (Figure 11).

The bioreactor reuses the cell suspension for TCE degradation. Thus, we designed batch studies that simulated multiple TCE exposure. A cell suspension was exposed to TCE, the enzyme activity measured and recovered and then reexposed to a second TCE dose. It was possible to degrade TCE and recover enzyme activity three to four times; however, the rate of recovery decreased after each TCE exposure. In these preliminary experiments, no additional nutrients were added except formate and methane. This could be one factor in the decreased recovery rates. A second factor could be the effect of headspace. These experiments used 100 mL liquid and 150 mL headspace. Previous experiments (described above) indicate that headspace volume can affect the stability of enzyme activity.

4. "Protectant" Chemicals/Free Radical Scavengers and sMMO Activity

Initial experiments determined that all protectant chemical facilitated recovery of sMMO activity (Figure 12). The mode of action of these protectant chemicals was not specifically identified. We did note that the addition of the protectant chemicals after TCE degradation did not facilitate recovery as well as their addition during TCE degradation. We also identified a slight lag in TCE degradation with the addition of these compounds.

D. DISCUSSION

1. Reduction in sMMO Activity

The loss of sMMO activity with the removal of methane and exposure to TCE was quite pronounced and was consistent with most previously published data (e.g., Alvarez-Cohen and McCarty, 1991). Strandberg *et al.* (1989) reported that the absence of methane for four hours was sufficient to halt TCE degradation in trickle filter bioreactors exposed to air. In experiments with methane starved cells, Jansen *et al.* (1991) found that the half-life of TCE degradation capacity in the absence of methane was 2.7 hours and that addition of formate to provide a reductant did not overcome this loss of activity.

The oxidation of TCE is apparently detrimental to methanotrophs and results in loss of TCE degradation capacity (Oldenhuis et al., 1991). This toxicity is in part likely due to the inhibition of the sMMO by the TCE epoxide. Toxicity is much lower for cells grown on methanol that do not express MMO than it is for cells grown on methane (Eng and Palumbo, 1991). Although some substrates, i.e., acetylene, appear to be suicide substrates (Oldenhuis et al., 1991) and specifically result in toxicity to methane oxidizing activity, there is evidence that TCE toxicity is non-specific (Oldenhuis *et al.*, 1991).

Although these experiments were not designed to distinguish between sMMO activity loss due to lack of methane and loss due to TCE oxidation there was evidence for a compound effect of the two conditions on sMMO activity. Recovery of the sMMO activity in treatments exposed to TCE and those only deprived of methane was different in rate and extent of sMMO recovery during the recovery periods (e.g., Figures 5-7, also see section below).

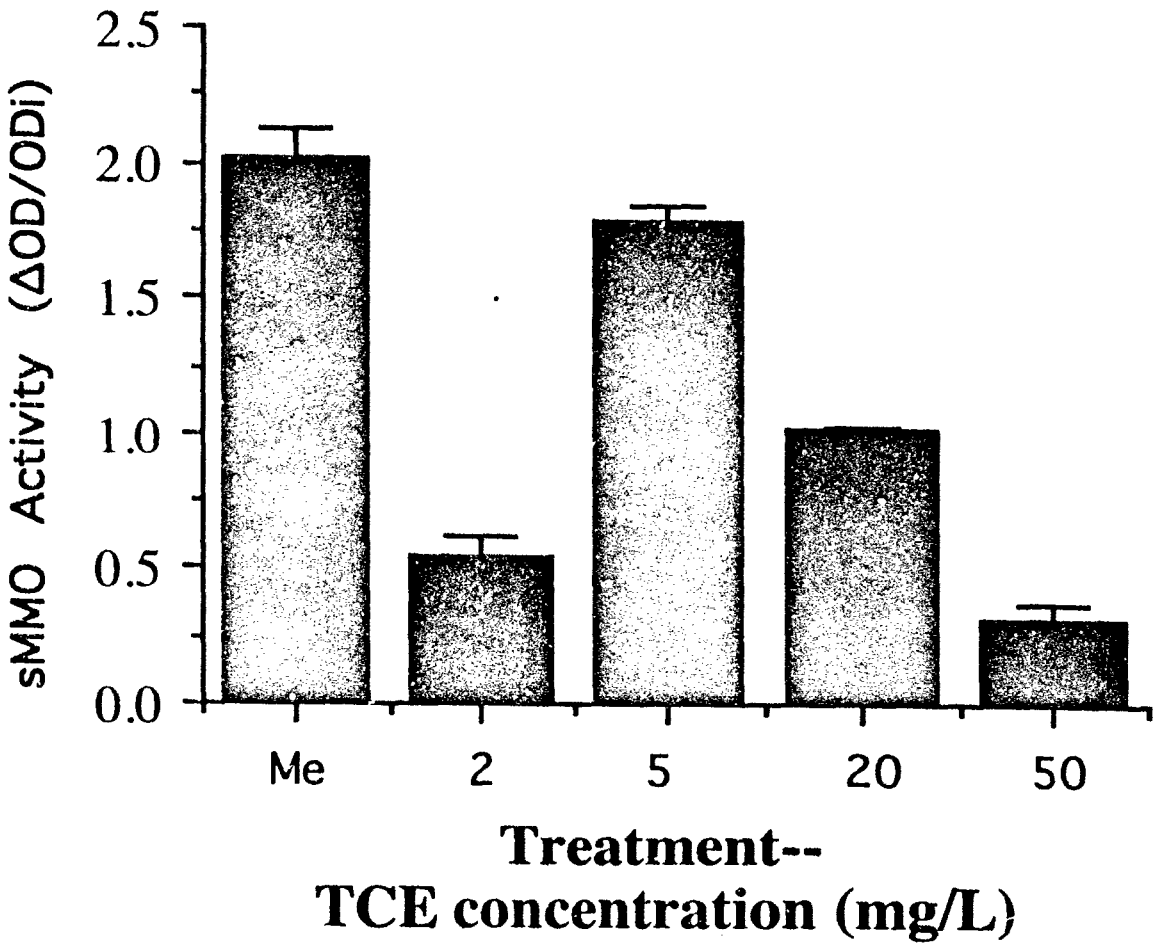

Figure 11. Effect of various TCE concentrations on the recoverability of sMMO activity (n=2). Enzyme activity reported as a change in OD/OD initial.

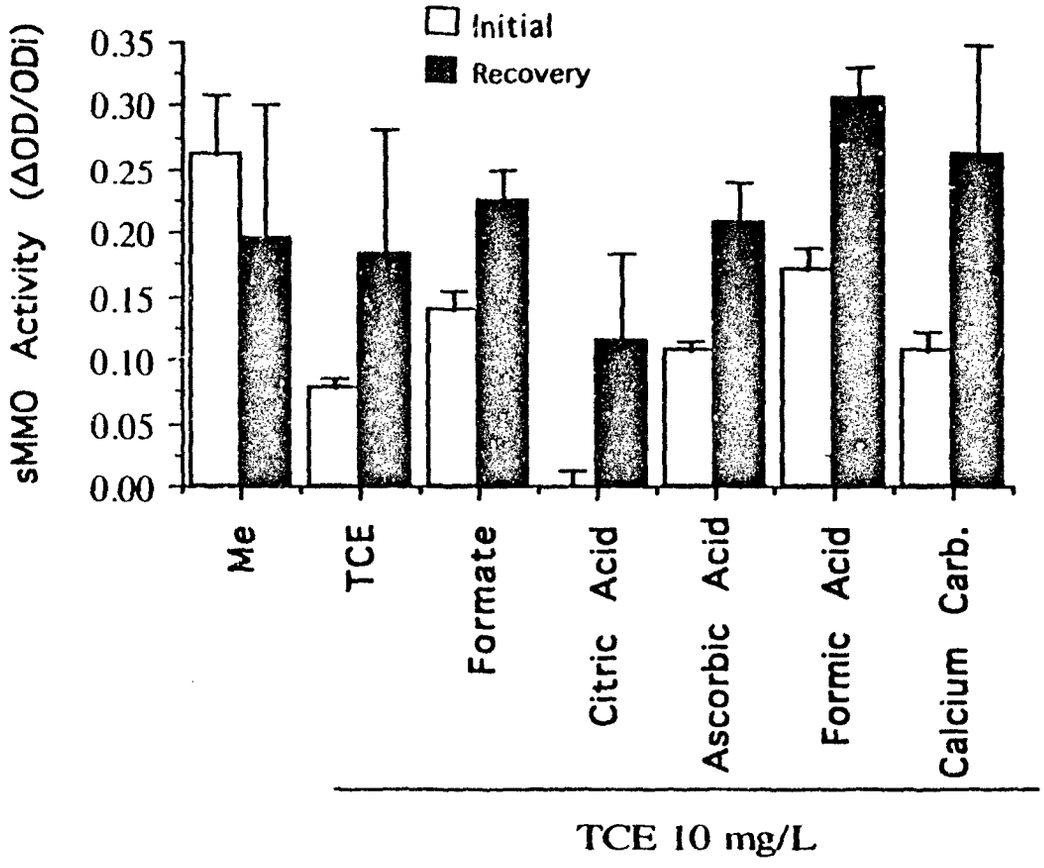

Figure 12. Effect of various protectant chemicals on the recoverability of sMMO after degradation of 10 mg/L TCE (n=2). Enzyme activity reported as a change in OD/OD initial.

The toxicity of TCE to cells has been documented by a variety of means including reductions in TCE degradation rates at high TCE concentrations. The data presented here indicate that even at relatively low TCE levels (1 mg/L) there is damage to metabolic systems that results in reduction in sMMO activity that is different from loss due to the absence of methane.

In many studies, the toxic effects of TCE have been linked to degradation rates (Janssen et al. 1987, 1988; Oldenhuis et al., 1989; Eng et al., 1991; Zylstra et al., 1989). Irreversible binding of the epoxides from chlorinated ethylenes to liver cytochrome P-450 has been demonstrated (e.g. Guengerich et al., 1979; Forkert et al., 1987; Forkert and Birch, 1989). While this enzyme differs from methane monooxygenase in some respects, both can generate the same epoxide structures from chlorinated ethylenes. Thus, the rate of epoxide formation may be critical in toxicity.

Based on the data presented here and on published data in the literature, we hypothesize that both short- and long-term effects of formate addition contribute to recovery of the sMMO levels in OB3b. In the short term, formate provides reducing power to the cells. In the long term, formate can represent an additional carbon source used to build cell biomass and under the right conditions (i.e., presence of methane) can eventually result in additional sMMO generation.

The effects of formate appear to be quite complex and, even as a source of reducing power, there may be two effects. One effect explored by other investigators is the additional supply of reducing power provided by formate for TCE degradation. In this mode, formate must be present with the TCE. However, there is a limit on how long the addition of formate will help. This limit may be the result of continued inhibition or destruction of the sMMO by the degradation of TCE. At this point, the second effect of formate addition may become more important. A substantial reduction in the level of sMMO activity should significantly reduce the ability of the culture to use methane for energy and carbon. This limitation will slow the recovery of the cells from the effects of the TCE. Since the flow of energy and carbon into the cell is reduced, production of new enzymes, including sMMO, and other cellular components would be limited. At this point, addition of formate can provide the cells with a source of carbon and reducing power that does not have to be mediated by sMMO activity. Thus, in the presence of methane, some of this new carbon and energy can be directed to sMMO production. Another portion of carbon and energy may go into repair of general cellular damage.

2. Recovery of sMMO Activity

The ability to recover sMMO activity is important in maintaining high rates of TCE degradation. In many batch studies of TCE degradation, cells are grown in the absence of TCE, subsequently exposed to TCE (e.g., Tsien et al., 1992), degradation rates are measured, and the experiment is terminated and the cells discarded or used for biomass measurements. Very high rates of TCE degradation can be demonstrated in these studies but the sustainability of the activity is not examined. Activity of sMMO is probably significantly reduced. However, our results suggest that it is possible to recover sMMO activity after TCE degradation there by saving the step of growing new biomass.

3. Effect of TCE Level

It is apparent from these results that inhibition of sMMO activity was more pronounced in the cultures exposed to TCE than in cultures that were only deprived of methane. Thus, exposure to increasing TCE levels results in slower recovery of both sMMO and more limited culture growth during recovery.

4. Effect of Formate

Several mechanisms result in the beneficial effect of formate on the recovery of sMMO activity. The effects of formate may be complex and nonlinear. The presence of formate does increase sMMO recovery but the highest levels of formate tested gave poorer results than the lower concentration tested. When the cells are damaged by TCE, much of the damage is probably to the sMMO enzyme although there may also be other cellular damage. The damage to the sMMO enzyme limits the ability of the cells to obtain energy from methane, since oxidation of methane to methanol by the sMMO enzyme is the first step in obtaining energy from methane. In a cell suffering from extensive damage to the sMMO enzyme, the rates of cellular material formation including new sMMO must be limited by the reduced activity of sMMO. Addition of formate can supply reducing power to the cells without requiring sMMO activity. Providing sufficient reducing power without adding an excess of formate may be the key. This should permit higher levels of activity within the cells including higher levels of protein synthesis.

The products of the increased protein synthesis are probably influenced by the concentration or amount of formate present. At high levels of formate, the cells apparently do not produce significant quantities of sMMO. Energy is available via the formate; thus, sMMO is not needed for cellular metabolism. The production of sMMO by the cells may not occur until formate is exhausted or is at a certain threshold level. Thus, at low levels formate should be exhausted sooner and thereby the cells could be stimulated into producing sMMO.

Long-term effects of formate on TCE degradation are likely achieved by other mechanisms, such as growth stimulation. In these short-duration experiments the effect of growth is probably present but is limited in scope.

5. Criteria for Enzyme Recovery

The criteria necessary for enzyme recovery in batch experiments were; the addition of formate at less than 4 mM; the addition of methane; the avoidance of copper and an initial biomass >0.5 OD to identify significant enzyme activity knockdown and recovery. Given these conditions, cells expressing high levels of sMMO degraded TCE (absence of methane) to below detection limits (<10 mg/L). Immediately following TCE degradation (or an overnight exposure to TCE) formate (<4 mM) and methane (<60 percent) were added to recover the greatest enzyme activity.

6. Application of Batch Experiments to the Recovery of sMMO Activity in the Bioreactor

All initial assay conditions necessary for enzyme recovery were determined in batch cultures. This information was used to determine initial operating conditions for enzyme recovery in the bioreactor. TCE feed rates and concentrations were chosen based on initial degradation experiments (0.2-2 mg/L). Formate levels were initially >10 mM and resulted in good enzyme recovery.

However, since there was carryover of formate throughout the bioreactor the formate levels were lowered to <4 mM. This reduction in formate concentration provided good enzyme recovery without carry-over of formate throughout the bioreactor and would result in cost savings.

Batch experiments determined that sodium formate increased the pH. Initial bioreactor experiments utilized sodium formate and the bioreactor pH also increased. To stabilize the pH, the recovery regime was modified to include formic acid instead of sodium formate and obtained recovery rates equal to sodium formate addition. The bioreactor test schedule did not permit other protectant chemicals to be evaluated for free radical scavenging effectiveness in the bioreactor.

These results and the literature suggest the potential for judicious use of formate to increase TCE degradation. Additional experimentation is needed to determine the optimal concentration of formate to be added and for what period it is necessary. Although this is likely to depend on the bioreactor configuration, TCE exposure, and other variables some general principles may become evident. These results indicated that lower concentrations can be used to achieve the desired results. Shorter exposure periods may work as well. Future areas for experimentation include defining the conditions that are likely to be useful in the bioreactor system presently being tested (Section IV), and evaluating the possibility of protecting the cells in the bioreactor from the effects of TCE epoxide formation.

SECTION IV

BIOREACTOR STUDIES

A. INTRODUCTION

Methylosinus trichosporium OB3b is a methanotroph capable of degrading TCE as a cometabolite through the enzyme soluble methane monooxygenase (sMMO), which is induced in copper-limiting conditions (Stanley et al., 1983). In the presence of copper, sMMO synthesis is suppressed, while the synthesis of particulate (membrane-associated) methane monooxygenase is promoted. It has been found that the conversion products of TCE degradation (epoxide or acylchlorides produced by hydrolysis) react with the sMMO hydroxylase component, rendering it inactive for further degradation (Ensley, 1991).

It has been shown previously that the sMMO of *Methylosinus trichosporium* OB3b degrades TCE at a much higher rate (V_{max} = 200 nmol TCE/min/mg cells; K_s = 137 μM) (Oldenhuis et al., 1990) than pMMO (V_{max} < 0.5 nmol TCE/min/mg cells) (DiSpirito et al., 1992). Several bioreactor designs for the degradation of TCE have been studied including attached-film, expanded-bed and fluidized-bed systems (Fogel et al., 1987; Strandberg et al., 1989; Phelps et al., 1991; Strand et al., 1991; Fennell et al., 1993) and two-stage dispersed-growth bioreactors (Alvarez-Cohen and McCarty, 1991; McFarland et al., 1992). Problems persist with copper suppression of sMMO, reduction of TCE degradation levels due to methane competition, and TCE byproduct toxicity (Alvarez-Cohen et al., 1991). To alleviate some of these problems a two-stage dispersed-growth bioreactor was proposed and modelled by Alvarez-Cohen and McCarty (1991a, 1991b). This system discards spent cells after contact with TCE. Under optimal conditions, it was hypothesized that 53 μmol of methane will be needed to replace cells inactivated by 1 μmol of TCE (Alvarez-Cohen and McCarty, 1991). Ideally, in order to optimize the efficiency of this system, a low proportion of the biomass should be discarded, and the regeneration of inactivated cells should be achieved. A degree of cell wastage is required, not only to simplify the construction and operation of the system, but also to keep the biomass actively growing, thus maintaining sMMO activity.

The objective of this research was to design a bioreactor around the biology of methanotrophic sMMO production to greatly enhance the efficiency of TCE degradation. In order to fully utilize methanotrophs for TCE degradation, TCE contacting and degradation were uncoupled from cell growth and sMMO production, reducing competitive inhibition. Additionally, the rate of mass transfer of methane, oxygen, and nutrients to the cells were increased by utilizing the cells in an aqueous suspension, rather than by fixed-film. Methane-limiting conditions for methanotroph growth was avoided by operating the reactor system under pressure to increase gaseous oxygen and methane mass transfer due to greater liquid-phase gas solubility according to Henry's Law.

Originally, it was planned to use the strain *Methylosinus trichosporium* OB3b (= ATCC 35069) in all experiments. Initial bioreactor experiments indicated the presence of a low level of copper (~1 μM) was interfering with sMMO activity of the strain. To alleviate this problem, a copper-tolerant mutant of *Methylosinum trichosporium* OB3b was utilized in all bioreactor experiments. The mutant (designed PP358; obtained from Dr. George Georgiou, Dept. of Chemical Engineering, the University of Texas at Austin) (Phelps et al., 1992) is identical to the wild-type except that its ability to metabolize copper has been disabled (Fitch et al., 1993).

B. METHODS AND MATERIALS

1. Bioreactor Design Considerations

In order to reduce competitive inhibition between methane and TCE, a bioreactor was developed to separately compartmentalize cell growth and sMMO synthesis from TCE contacting and degradation. The first unit included a pressurized CSTR (continuous stirred tank reactor), in which cells were continuously cultivated with methane and oxygen. A recycle loop through a cellular filtration column allowed water, filtered from cells, to leave the system, and recycled active biomass back to the CSTR. The second section consisted of a series of four plug-flow contact columns, in which methane-limiting conditions exist and TCE degradation occurs. A flowsheet of the designed system with numbering of discrete sampling points and flows in the system is shown in Figure 13. The system was designed to have no gaseous head-space, eliminating problems associated with stripping of TCE to the gas phase, and thus allowing accurate mass balances to be determined. The TCE contact columns were made in a series to allow versatility for future experiments, additions and modifications, and to allow easy manipulation to vary residence times. In addition, the placement of discrete sampling points allowed monitoring throughout the length of the TCE contact columns for accurate analysis of the degradation kinetics of the system.

2. Detailed Bioreactor Design

The bioreactor system (Figure 14) consists of a continuous stirred tank reactor (CSTR), a series of plug-flow reactor columns, and a dewatering system. All components of the system were composed of stainless steel, glass, Teflon®, or viton rubber, in order to minimize adsorption of TCE or adhesion of biofilm to the bioreactor surfaces. Tube fittings were either NPT or Swagelok® (Swagelok Co., Solon, Ohio), and flanged surfaces were machine finished and sealed with o-rings. The system has been pressure tested to 11.2 atm, without leakage, but normal operating pressure is in the range of 1 to 4 atm.

a. Continually stirred tank reactor (CSTR)

The CSTR is a full-liquid volume 2.4-L 316 stainless steel unit containing two six-bladed downward thrust agitators and a six-bladed radial agitator (Figure 15) connected to a Permanent Magnet DC motor with SRC control (Dayton Electric MFG., Co., Chicago, Ill.). Pressure control for the entire system occurs in the CSTR, via a variable tension, spring-loaded backpressure relief valve (Escom Corp., Elk River, Minn.), allowing undissolved gases to escape from the system to a vent, and also allowing excess liquid to self-regulatingly escape to a waste tank, as a back-up to the dewatering device. Dissolved oxygen concentration in the CSTR is measured by a stainless steel oxygen probe (Ingold Electrodes Inc., Wilmington, Mass.) connected to a dissolved oxygen meter (Cole Palmer, Chicago, Ill.). A glass window in the CSTR allows the liquid level to be monitored visually.

A mechanical seal, consisting of a ceramic seat and a graphite head is used to maintain a gas tight seal around the agitator shaft as it exits the CSTR. A thrust bearing pressed into the mechanical seal shroud allows minimal friction surface for the agitator shaft to press against, while

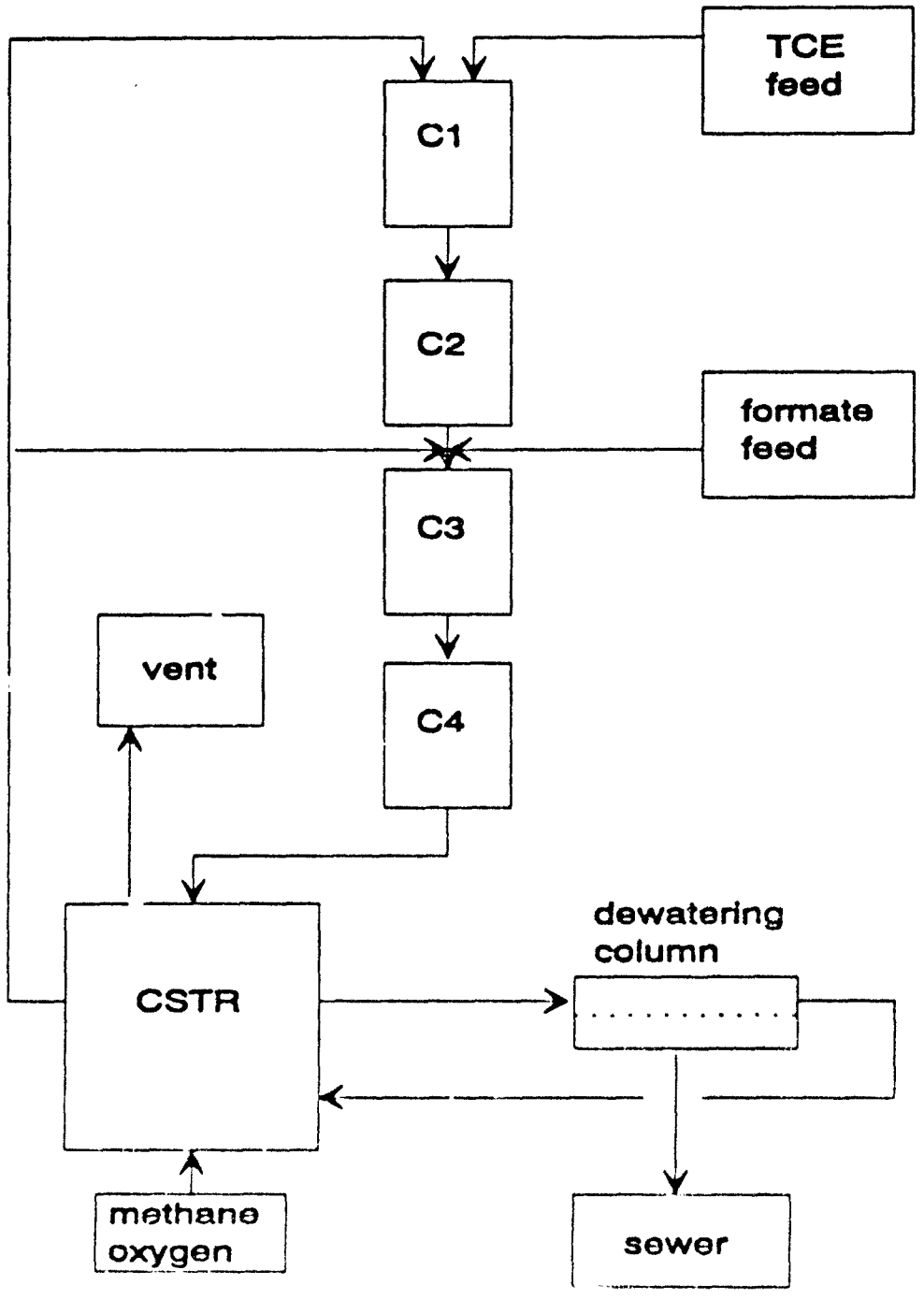

Figure 13. Simplified schematic diagram of bioreactor system equipped for formate addition during cross-flow operation. Cell addition between Columns 2 and 3 was not used in the once-through operational mode.

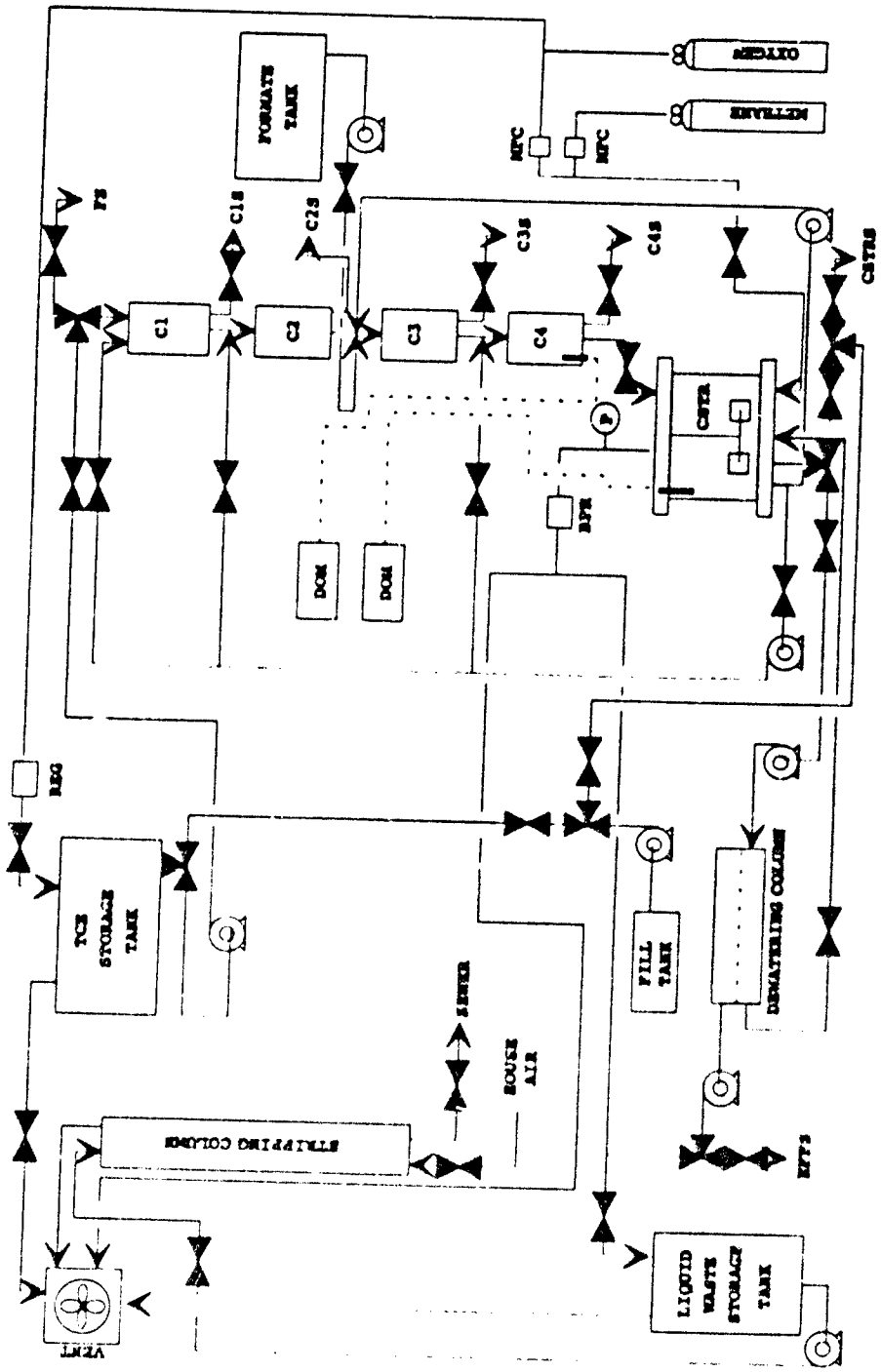

Figure 14. Piping diagram of bioreactor system equipped for formate addition during cross-flow operation.

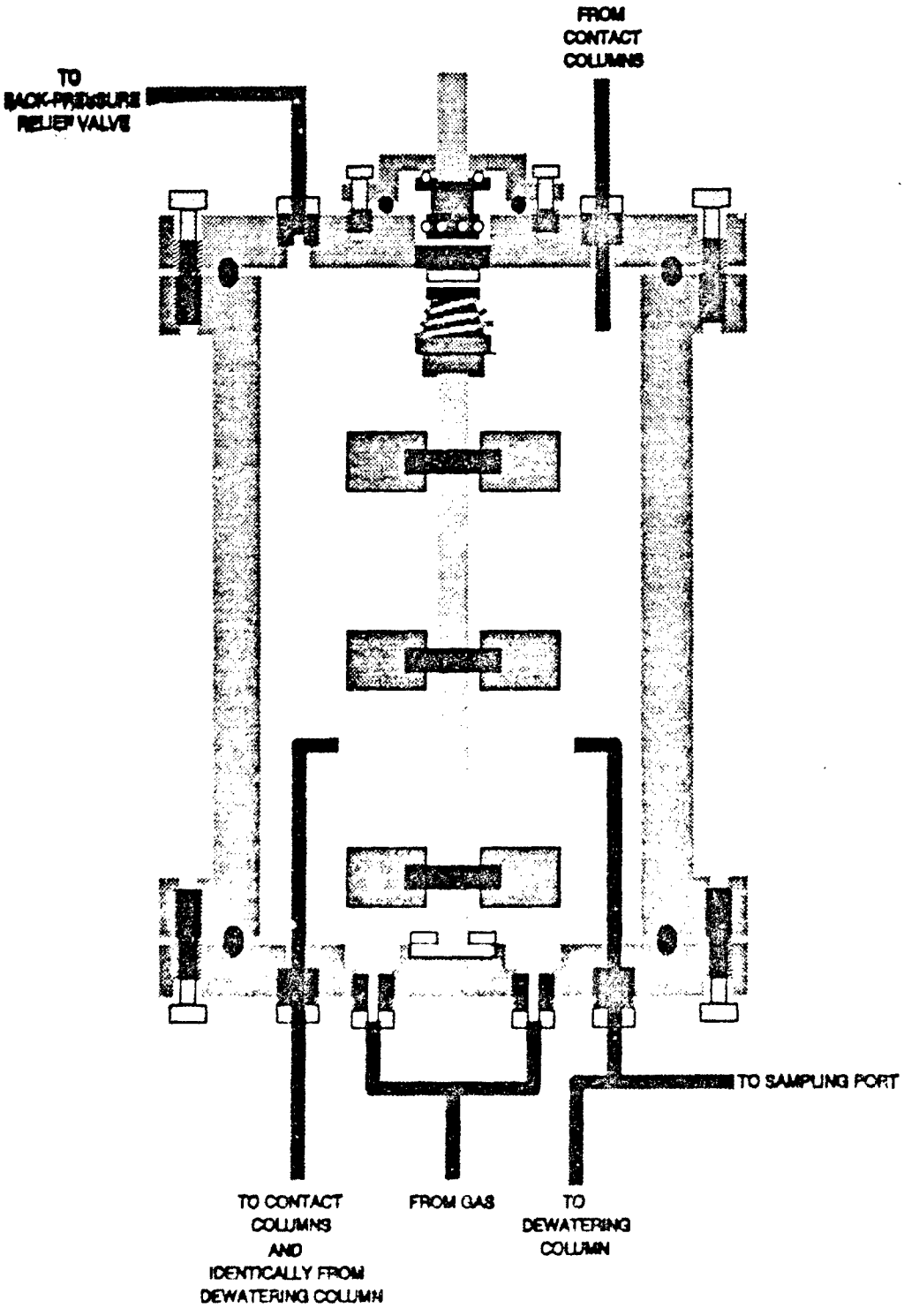

Figure 15. Diagram of the continually stirred tank reactor (CSTR) component of the bioreactor system.

providing a contacting force between the head and the seat of the seal. A radial bearing in the mechanical seal shroud, and a teflon bushing in the base of the CSTR allow pivot-free rotation of the shaft. A universal joint in series between the agitator motor and the shaft allows the shaft to remain vertical during small alignment variations of the agitator shaft and the CSTR.

Methane and oxygen are introduced into the CSTR through two Model 8272 mass flow controllers coupled to a Model 8284 dynablender (Matheson Gas Products, Montgomeryville, Penn.). The gases were mixed in a manifold and entered the CSTR through a porous metal diffuser, located at the bottom of the CSTR. Check valves between the mass flow controllers and the manifold prevent liquid from backing into the controllers if pressure is reduced upstream, below the reactor pressure.

b. Plug-flow reactor series

Media and cells are pumped through 0.32 cm (1/8") O.D. (outside diameter) stainless steel tubing from the CSTR to a vertical series of four 6.35 cm I.D. (internal diameter) stainless steel pipe TCE contacting columns through either or both variable speed FMI model QV metering lab pump with an FMI Model V100 stroke rate controller (Fluid Metering Inc., Oyster Bay, N.Y.) (one leads to Columns 1, 2, and 4 and the other leads to Column 3). Valves allow control of flow from the CSTR to any one of the four columns (Figure 14). The volume of the TCE contacting columns, from the top column to the bottom column, are 0.57 Liter, 0.81 Liter, 0.89 Liter, and 1.02 Liters. The columns are connected to one another in series by 0.64 cm (1/4") O. D. stainless steel tubing. A stainless steel dissolved oxygen probe (Ingold Electrodes Co.) connected to a dissolved oxygen meter (Cole-Palmer) is located in the lowest column for measuring the oxygen concentration after contact of the biomass with TCE. The TCE contacting columns empty into the CSTR with 0.64 cm O. D. stainless steel tubing for cell recycle.

c. Synthetic TCE feedstock

Synthetically TCE-contaminated water was prepared in a 19-Liter stainless steel carboy (Gelman Filtration Products, Ann Arbor, Mich.) and then was pumped through a centrifugal pump (Cole-Palmer) to a stainless steel storage tank under a 1.7 atm oxygen blanket for subsequent addition to the bioreactor. The TCE solution was delivered to the top of the uppermost TCE contacting column through a variable speed FMI model QV metering lab pump with an FMI Model V100 stroke rate controller (Fluid Metering Inc.) through 0.32 cm O.D. stainless steel tubing.

d. Dewatering device

To maintain a constant volume of liquid in the bioreactor and to prevent cells from escaping with the effluent stream, a tangential flow dewatering device with feed-side recycle was used. Liquid was recycled from and to the CSTR through a 6.35 cm I.D. stainless steel pipe, 30.5 cm in length, using a variable speed QD metering lab pump with a stroke rate controller (Fluid Metering Inc.). Another similar pump delivers the bioreactor effluent stream from the CSTR through a 3.81-cm O.D. porous metal filter, 15.2 cm, in length which was covered with 0.22 μm pore-size Teflon® (Millipore). The filter was located inside the stainless steel pipe. The effluent stream after filtration passed to a waste storage tank.

e. Waste storage tank and stripping column

Liquid accumulating in the waste storage tank was pumped through a Slo-Syn metering pump (Superior Electric Co., Bristol, Conn.) to the top of a packed-bed, trickle-filter stripping column for final TCE removal (TCE not degraded by the bioreactor). The stripping column contained ceramic saddles as packing material. House air was then used to strip the TCE waste liquid with gas vented to the outside, while effluent liquid was gravity fed to the drain.

3. Routine Bioreactor Operation

a. TCE feedstock and growth medium

The bioreactor system was filled with a modified nitrate mineral salts medium (NMS; Cornish et al., 1984) (Table 5). The medium was prepared with deionized water (resistivity >15 MOhm). The TCE feedstock was prepared with NMS media and a TCE saturated water solution (8.31 mM at 25°C) (Verschueren, 1983) to a specified TCE concentration.

b. Bioreactor culture and inoculation

Methylosinus trichosporium OB3b mutant PP358 was kindly provided by Dr. George Georgiou of the University of Texas, Austin, TX. This strain was cultivated either in liquid or on agar-solidified NMS media (Table 1), under a 1:4 methane:air headspace, at 28°C for 7 days. The CSTR was inoculated with 1 Liter of PP358 culture (A_{600} ~ 0.5), and NMS media was then added to fill the CSTR and the remaining sections of the bioreactor completely. The CSTR agitator was rotated at 138 rpm. Cells were recycled from the CSTR to the top of Column 1 at a rate of 10 mL/min. Methane and oxygen were initially added at 10 mL/min each, and the flow rates were altered as the bioreactor biomass increased. The oxygen concentration was manually kept around 5-15 percent saturation at 3 atm (1.4 - 4.2 mg/L). The bioreactor internal pressure was set at 3.0 atmospheres by adjusting the back pressure relief valve. PP358 was recirculated throughout the bioreactor in the absence of TCE addition until it had reached stationary growth phase.

c. Sample acquisition

Valved liquid-phase sampling ports (leading to 16-gauge needles) are located at the bottom of the CSTR, at the bottom of each of the TCE contacting columns, from the simulated TCE wastewater feed line, and in the dewatering line prior to deposition into the waste tank. Five-mL liquid bioreactor samples were obtained into screw cap septum vials sealed with Teflon®-lined silicon seals (32 mL; Pierce, Rockford, IL).

4. Analytical Procedures

a. Biomass determination

PP358 culture optical density was determined spectrophotometrically by measuring absorbance at 600 nm using a Beckman DU-70 spectrophotometer (Beckman Instruments, Fullerton, CA) and comparison of optical density of the sample to a standard curve of optical density vs. dry weight (mg/L).

TABLE 5. FORMULATION OF THE NITRATE MINERAL SALTS MEDIUM USED IN ROUTINE BIOREACTOR OPERATION.

Constituents:	Final concentration	Constituents	Final concentration
$NaNO_3$	2 mM	Bacto-yeast extract	0.025% (w/v)
phosphate buffer (pH 6.8)	2 mM	cyanocobalamin	0.4 µg/L
$MgSO_4.7H_2O$	150 µM	Ca pantothenate	20 µg/L
ferric EDTA	50 µM	niacinamide	20 µg/L
$CaCl_2.2H_2O$	50 µM	thiamine-HCl	20 µg/L
$CuSO_4.6H_2O$	5 µM	riboflavin	20 µg/L
$MnSO_4.4H_2O$	2 µM	d-biotin	8 µg/L
$ZnSO_4.7H_2O$	2 µM	folate	8 µg/L
H_3BO_3	2 µM	*p*-aminobenzoate	8 µg/L
K_2SO_4	1 µM	pyridoxine	8 µg/L
KI	1 µM	L-ascorbate	8 µg/L
$CoCl_2.H_2O$	0.65 µM	pyridoxamine	4 µg/L
$Na_2MoO_4.2H_2O$	0.4 µM	pyridoxal	4 µg/L

b. Soluble methane monooxygenase (sMMO) activity

The sMMO activity was determined by a modification (Hoh et al., 1993) of the naphthalene oxidation assay (Brusseau et al., 1990), whereby naphthalene is oxidized by sMMO to form naphthol (naphthalene is not oxidized by pMMO). The sMMO activity was normalized by cell dry weight. sMMO activity is expressed as nmol (naphthol formed)/hour/mg cells. Naphthol formation is linear over the 1-hour period and can be regarded as a specific rate.

c. Trichloroethylene analysis

TCE levels were calculated by gas chromatographic analysis of vial headspace samples. Analysis was performed on a Shimadzu GC 9AM gas chromatogram (Shimadzu Analytical Instruments Co., Kyoto, Japan) equipped with a split injector port operated at 250°C, a 60 m x 0.53 mm I.D. R_{TX} volatiles capillary column (Restek Corp., Bellefonte, Penn.) operated isothermally at 120°C, and an electron capture detector operated at 300°C. Nitrogen was used as the carrier gas (30 mL/min). The peak areas were integrated with a Shimadzu C-R6A Chromatopac. The bioreactor liquid sample TCE concentration was determined by Henry's Law (Gosset 1987) with normalization of chromatographic peaks to a uniform ratio of gas- and liquid-phase volume (27 mL: 5 mL).

d. Methane and oxygen analysis

The concentration of methane in samples was determined by analyzing head space samples containing methane by gas chromatography using a Shimadzu GC 9 AM chromatogram, equipped with a flame ionization detector and a 15m x 0.53 mm I.D. AT-1 capillary column (Alltech, Deerfield, IL) maintained at 60°C using nitrogen as the carrier gas (1 mL/min). The liquid bioreactor sample methane concentration was then determined by Henry's Law (Atkins, 1986) with normalization of chromatographic peaks to a uniform ratio of gas- and liquid-phase volume (27 mL: 5 mL). Oxygen concentrations were determined by percentage of saturation of oxygen from the oxygen probe readout. Saturation concentrations of oxygen were then calculated empirically.

e. Additional analyses

Analysis of the bioreactor culture was undertaken to determine methanotroph populations in the nonaseptic conditions utilized. Bioreactor samples were serially diluted onto R2A and nutrient agar and incubated 7 days at 28°C. Representative colonies were picked and transferred to nutrient agar and purified. The subsequent nine isolates were identified using API NFT, API 20E (API Laboratory Products, Inc., St. Laurent, Quebec, Canada) and Biolog. identification test kits (Biolog Inc., Hayward, Cal.).

On occasion, analysis of the off-gas from the CSTR was performed. Due to the relative difficulty and inconvenience, manual sampling was only done to ascertain the level of off-gas methane and TCE in the second of the abiotic experiments. Gas was collected in a 60 mL syringe through a viton rubber tube and then transferred into valved aluminized gas sample bags (Gas Calibration Instruments Co., Ardsley, N. Y.). The methane and TCE in the gas samples were then sampled with a 50 µl gas-tight syringe and analyzed by gas chromatography as explained above. Formate levels in bioreactor samples were analyzed by HPLC as detailed in Section III. Before analysis cells were removed by centrifugation.

Feedstock constituents were also on occasion analyzed to determine if nutrient limitation was occurring. The components analyzed included nitrate, phosphate, iron, and magnesium. Methods for analysis of these compounds is presented in Section II.

5. Abiotic Experimentation

A series of abiotic TCE mass balance experiments were performed for the system. These experiments served a number of purposes. The first purpose was to determine if significant loss of TCE was occurring through leakage and adsorption to surfaces. Second, these experiments would determine how long the reactor would take to attain a "steady-state" level in which the reactor TCE concentration equalled the TCE feed concentration. This experiment would also indicate the degree of loss of TCE through the off-gas from the CSTR. Last, a control experiment was run in which cells were present in the reactor. These cells did not receive any methane for growth. This experiment would serve as a final check for mass balance closure as well ascertain relative losses of TCE through the off-gas and through cell adsorption.

a. Passive abiotic experiment

The first experiment involved filling the reactor completely with tap water and recycling the water through the columns and back into the reactor at 10 mL/min. Two separate TCE levels were examined. Saturated TCE was added to the water to bring the final reactor TCE concentration to 3 mg/L and 0.150 mg/L. The system was operated for 2 days at 3.0 atm for each experiment. Three replicate liquid samples were then taken at 0 and 48 hours for each experiment. Samples were analyzed for TCE content.

b. Abiotic experiment with continuous TCE addition

A TCE feed concentration of 1 mg/L (prepared in NMS medium) at a flow rate of 2.1 ± 0.1 mL/min was added to bioreactor. Liquid from the CSTR was recycled through the TCE contacting columns at 10 mL/min. The bioreactor was completely cell-free. TCE levels were monitored for the inlet stream, all four contacting columns, and the CSTR.

c. Control experiment with continuous TCE addition in the presence of "inactive" biomass

A TCE feed concentration of 20 mg/L (prepared in NMS medium) at a flowrate of 2.1 ± 0.1 mL/min was added to bioreactor. Liquid from the CSTR was recycled through the TCE contacting columns at 10 mL/min. The bioreactor contained active biomass from a previous experiment (see below). Methane addition was halted and air was introduced instead at the same rate. Oxygen concentration in the CSTR was maintained between 5-15 percent of saturation. The experiment was performed over 4 days. TCE levels were monitored for the inlet stream, all four contacting columns, and the CSTR. Biomass and sMMO levels were also monitored.

6. TCE Degradation Experiments

a. Cell recycling modes

Cell recycling was operated in two different modes for the bioreactor TCE degradation experiments. The first mode, called the "single-pass" mode, involved cell recycle from the CSTR to the top of the first TCE contacting column (C1, see Figure 13). The single-pass cell recycle flow rate was always maintained at 10 mL/min. In the second mode, called the "cross-flow" mode, involved recycle from the CSTR to the tops of the first and third TCE contacting columns (C1 and C3, see Figure 13). The cross-flow cell recycle flow rate was maintained at 5 mL/min to each of the two contacting columns. Flow rates were monitored and maintained by a FR4000 Series Flowmeter (Key Instruments, Trevose, Penn.).

b. Experimental parameters

The TCE feed was pumped into the top of the first contacting column at 2.1 ± 0.1 mL/min in all experiments creating an overall dilution factor of 0.02 h^{-1}. The effluent stream from the dewatering device was maintained between 1.0-2.0 mL/min. The remainder of the effluent was self-regulated through the back pressure relief valve, thus ensuring the bioreactor maintained a fully filled state.

In certain of the TCE experiments, formate was added after a significant inhibition of sMMO activity was observed (due to TCE toxicity). Formate was added at a rate of 0.1 mL/min at the top of the third TCE contacting column using a Model 314 metering syringe pump (Instrumentation Specialties Co., Lincoln, Neb.). Initially sodium formate was used at a concentration of 1.0 M. A marked increased in pH and accumulation of nonutilized formate, however, prompted a reduction in the concentration of added formate. The formate solution added was 0.2 M and the pH was adjusted to pH 4.0; this maintained bioreactor pH between 6.8 and 7.5.

Table 6 lists the general parameters of the various bioreactor TCE degradation experiments including: mode of operation, TCE feed concentration, time of operation, and operation when formate addition was utilized.

c. Single-pass experiments

Five experiments were performed with different TCE feed concentrations, ranging from 0.2 to 20 mg/L (Table 6). The runtime of these experiments varied (Table 6) however in all cases the reactor was allowed to reach a pseudo-steady-state in terms of TCE degradation. In these experiments samples were obtained from the inlet stream, all four contacting columns, the CSTR, and occasionally from the dewatering device effluent stream on a regular basis. The following parameters were determined in each experiment: biomass levels; TCE, methane and oxygen concentrations; and sMMO activity level.

TABLE 6. PARAMETERS OF THE VARIOUS BIOREACTOR TCE DEGRADATION EXPERIMENTS

Mode of Operation	TCE Feed Concentration (mg/L)	Time of Operation (hours)	Formate Addition (conc., flow rate)
Single-Pass	0.2	432	
	1	408	
	3	90	
	10	216	0.2 M, 0.1 mL/min
	20	312	0.2 M, 0.1 mL/min
Cross-flow	1	120	
	3	168	
	10	192	1.0 M, 0.1/ mL/min

d. Cross-flow experiments

Three experiments were performed with different TCE feed concentrations, ranging from 1 to 10 mg/L (Table 6). The runtime of these experiments varied (Table 6) however in all cases the reactor was allowed to reach a pseudo-steady-state in terms of TCE degradation. In these experiments samples were obtained from the inlet stream, all four contacting columns, the CSTR, and occasionally from the dewatering device effluent stream on a regular basis. The following parameters were determined in each experiment: biomass levels; TCE, methane and oxygen concentrations; and sMMO activity level.

C. RESULTS

1. Bioreactor Cultivation Conditions and Characteristics

With the dewatering device operating; a dilution rate of 0.02 h^{-1}; a CSTR liquid methane and oxygen concentration at 3-5 and 1-3 mg/L, respectively, and a temperature averaging 22°C; biomass levels of 200-400 mg/L was regularly maintained throughout the reactor. Cell growth rates were found to range from 0.074 - 0.125 mg/L/min. The amount of residual methane and oxygen in the bottom of the last TCE contacting column was found to range from 0-0.9 mg/L and 0-0.2 mg/L, respectively. Since 80-90 percent of methane was lost as off-gas through the back pressure relief valve, the resultant cell yield ranged from 0.26 - 0.32 mg cells/mg methane.

Though the system was maintained nonaseptically in all experiments the background growth levels of contaminants never exceeded 10 percent of the total microbial community. Cell counts determined on R2A medium indicated 0.8-3.0 x 10^7 non-methanotrophic cells/mL compared to a total population equal to about 1-3 x 10^8 cells/mL (as indicated by calculated dry weight levels). No contaminants able to grow on methanol or methane were observed. The contaminants were mostly growing on the yeast extract component of the feedstock and were almost entirely non-methylotrophic Gram-positive bacteria of airborne origin, i.e., *Curtobacterium*, *Aureobacterium*, *Arthrobacter*, and *Pimelobacter*.

2. Abiotic Experiments

a. Passive abiotic experiment

This experiment demonstrated that the reactor held pressure for at least 48 hours and that TCE loss, due to stripping or adsorption was negligible. The first experiment used an initial TCE concentration of 3.13 ± 0.07 mg/L. After 48 hours, 3.05 ± 0.11 mg/L of the TCE was recovered. The second experiment used a lower initial TCE concentration of 0.123 ± 0.010 mg/L. After 48 hours, analysis revealed that 0.126 ± 0.007 mg/L was still present in the reactor.

b. Abiotic experiment with continuous TCE addition

Using reactor residence times based on designated flow rates, and treating the bioreactor system as 5 CSTRs in series, the theoretical value for which steady-state should be obtained was 130 hours (96 percent of fed TCE concentration in the effluent). Figure 16 shows the results of an actual empirical study to determine this for the bioreactor. The variations in feed concentrations was due

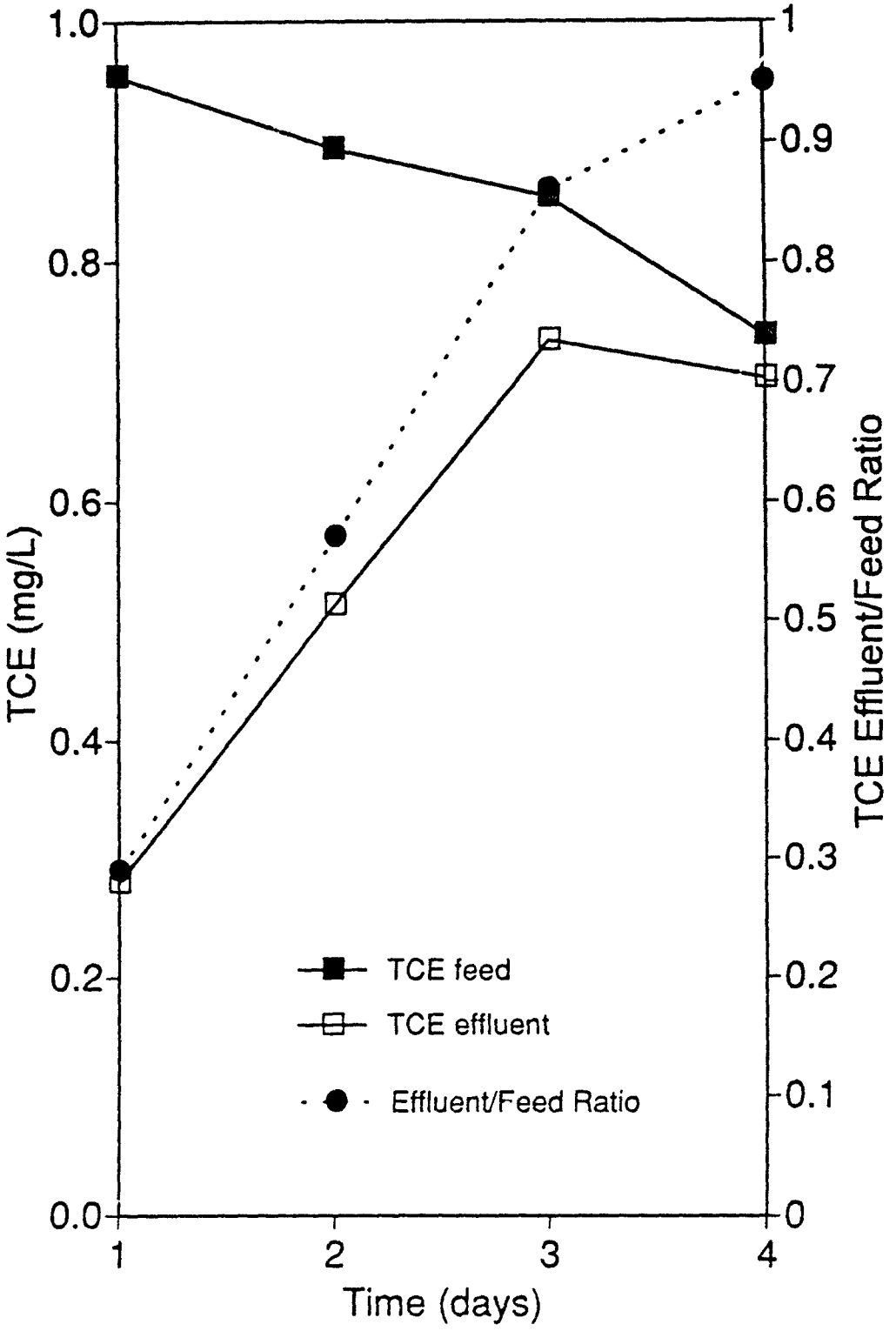

Figure 16. Abiotic experiment with continuous TCE addition. No biomass is present in the bioreactor.

to partitioning of the TCE with increasing headspace in the feed tank. A steady-state was approached around 96 hours, where the concentrations throughout the reactor converged with the TCE feed concentration. This was somewhat less than what was predicted. If an assumption was made that the reactor operated completely as a series of plug-flow reactors, it should theoretically take only 8 hours for the system to come to steady-state with the feed. The results thus indicate the bioreactor system behaves somewhere between the responses expected for an ideal CSTR and ideal plug-flow system.

c. Control experiment with continuous TCE addition in the presence of "inactive" biomass

At the completion of the 20 mg/L TCE single-pass experiment (see below) a control experiment was performed in which sMMO activity was deactivated by the exclusion of methane. Figure 17 shows the result of this experiment. Rapid increases in TCE levels were observed throughout the reactor with corresponding declines in the overall TCE degradation level. Due to lack of methane and the limitations of the dewatering device the biomass began to wash out. Complete methanotroph washout occurred after 72 hours. No sMMO activity was detectable in samples after only 48 hours. From this it can be concluded that absorption to biomass had a negligible effect on the TCE mass balance, and that TCE degradation is being carried out by the methanotroph population cultivated in the bioreactor.

3. Single-pass TCE Degradation Experiments

a. 0.2 mg/L TCE Feed

In this experiment complete degradation of the 0.2 mg/L TCE feed was attained during the entire experiment which lasted for 432 hours (18 days) (Figure 18). This occurred regardless of the variations in feed levels which ranged from a low of 0.14 ± 0.02 mg/L to a high of 0.23 ± 0.02 mg/L. TCE was completely degraded by the end of the third contacting column. During this experiment biomass rapidly declined from a level of 500-600 mg/L but stabilized within 4 days, averaging 240 mg/L. The sMMO activity of the cells in this experiment remained relatively stable ranging from 54 ± 6 to 67 ± 4 nmol/h/mg cells for the course of the experiment. The 10-20 percent increase in sMMO activity towards the end of the experiment was not considered statistically significant. No TCE toxicity effects were discernable. The maximal degradation rate was calculated to be 0.08 mg TCE/L/d.

b. 1 mg/L TCE Feed

In this experiment an average 94.5 percent ± 1.7 percent degradation of the 1 mg/L TCE feed was attained during the experiment which lasted for 408 hours (17 days) (Figure 19). Feed levels ranged from a low of 0.49 ± 0.01 mg/L to a high of 0.93 ± 0 02 mg/L, however degradation levels did not very significantly. TCE effluent levels measured in the CSTR averaged 39 ± 13 µg/L, however these levels were lower, averaging 19 ± 14 µg/L, after passing through the dewatering device possibly due to a degree of biological degradation. In this experiment biomass, as in the previous experiment, declined rapidly for the first 3 days (from an initial level of 600 mg/L) and then stabilized at 360 ± 30 mg/L after 4 days. The sMMO activity of the cells in this experiment also remained relatively stable ranging from 52 ± 5 to 64 ± 4 nmol/h/mg cells for the course of the experiment. The 10-15 percent decline in sMMO activity during the experiment is not considered statistically significant thus no TCE toxicity effects were discernable. The maximal degradation rate was calculated to be 0.62 mg TCE/L/d.

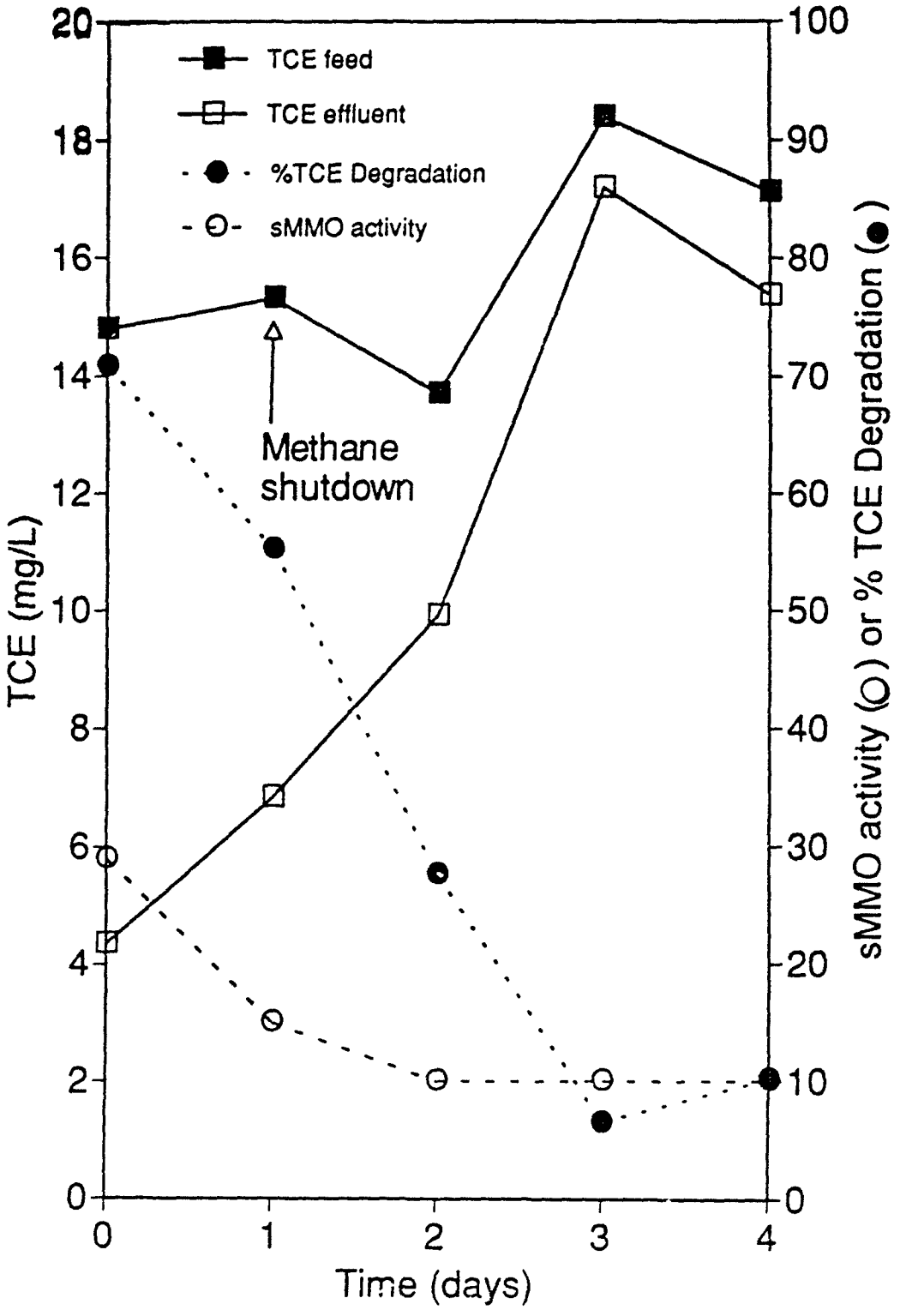

Figure 17. Control experiment in which methane shutdown occurred with replacement with air.

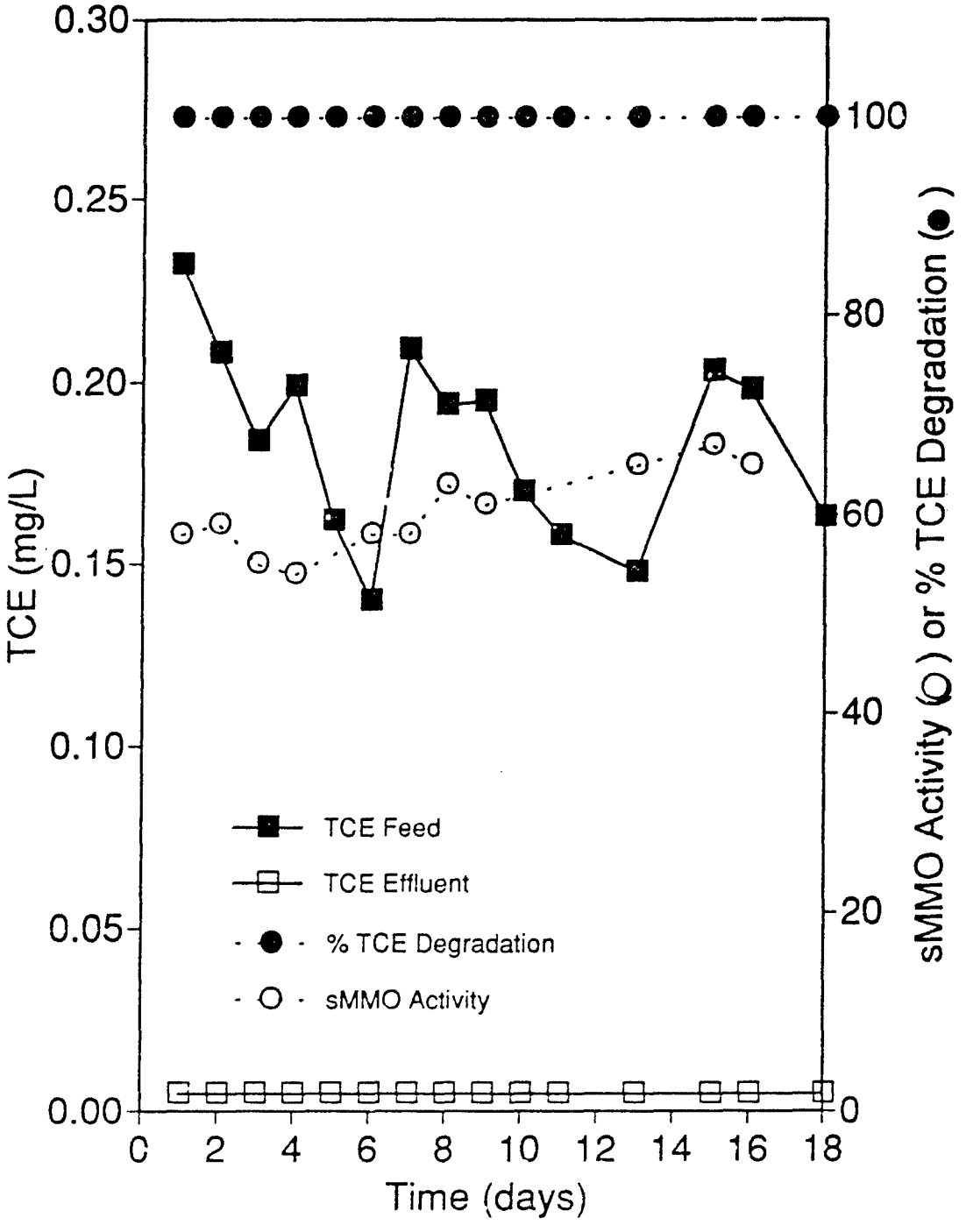

Figure 18. Single-pass experiment using a nominal TCE feed concentration of 0.2 mg/L.

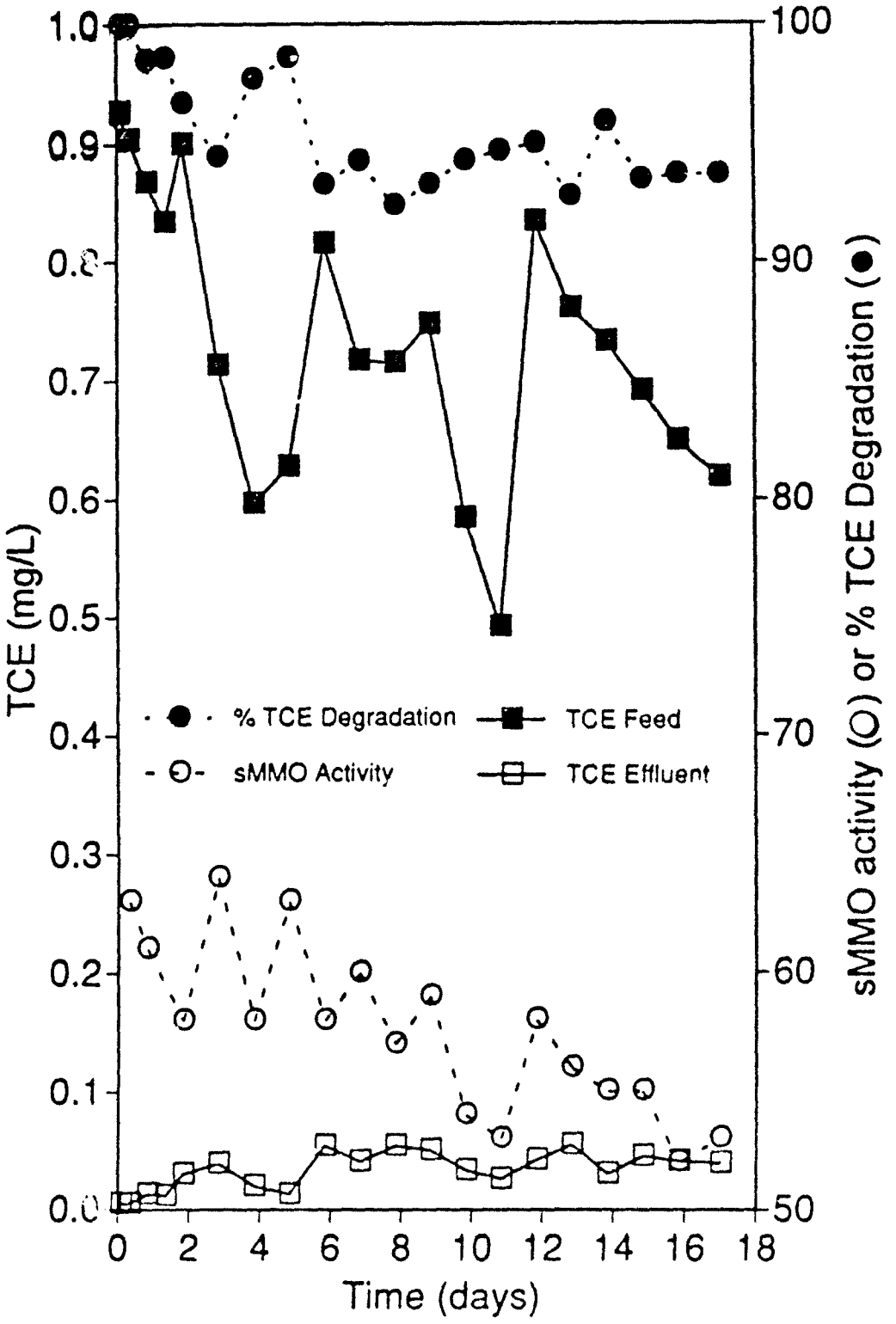

Figure 19. Single-pass experiment using a nominal TCE feed concentration of 1.0 mg/L.

c. 3 mg/L TCE Feed

In this experiment an average 83.9 percent ± 3.2 percent degradation of the 3 mg/L TCE feed was measured; however, this experiment only lasted 90 hours (Figure 20). The time needed for the reactor to reach a pseudo steady-state was not positively obtained. Feed levels ranged from a low of 2.34 ± 0.17 mg/L to a high of 2.73 ± 0.54 mg/L. TCE effluent levels measured in the CSTR at 90 hours was 0.46 ± 0.07 mg/L. In this experiment biomass stabilized at 320 ± 35 mg/L between 56 and 90 hours. The sMMO activity of the cells in this experiment remained relatively stable, ranging from 63 ± 4 to 71 ± 3 nmol/h/mg cells for the course of the experiment. No TCE toxicity effects were discernible. The maximal degradation rate was calculated to be 3.31 mg TCE/L/d.

d. 10 mg/L TCE Feed

An average 83.4 percent ± 3.1 percent degradation of the 10 mg/L TCE feed was determined in this experiment which lasted 216 hours (9 days) (Figure 21). Feed levels ranged from a low of 7.28 ± 0.11 mg/L to a high of 9.85 ± 0.55 mg/L. The sMMO activity of the bioreactor biomass was determined to be 64 ± 3 nmol/h/mg cells before TCE introduction commenced. After the TCE feed was introduced a reduction in sMMO levels in the bioreactor were observed. Within 4 days the sMMO activity level in the bioreactor had fallen to 42 ± 7, a 34 percent decline. This significant decline in sMMO activity indicated a demonstrable TCE toxicity effect was occurring. Formate additions thus commenced (see Section III for rationale), with 0.2 M formate (pH 4.0) added at 0.1 mL/min. Formate caused a slow rise in sMMO activity levels which by 9 days had increased to 65 ± 2, equivalent to the original activity level. The pH of bioreactor samples did not exceed 7.5. After 4 days of operation the TCE degradation levels was only 78.1 percent, however with formate addition the level had increased to 84-85 percent. TCE levels in the effluent averaged 1.37 ± 0.14 mg/L. In the experiment biomass eventually stabilized at 280 ± 30 mg/L.

e. 20 mg/L TCE Feed

With a 20 mg/L TCE feed overall degradation slowly declined during the progress of the experiment which lasted 312 hours (13 days) (Figure 22). Feed levels ranged from a low of 14.69 ± 1.82 mg/L to a high of 18.55 ± 0.82 mg/L. The degradation levels after 4 days was approximately 80 percent however after 13 days this level had dropped to 71 percent. The sMMO activity of the bioreactor biomass was determined to be 74 ± 3 nmol/h/mg cells before TCE introduction commenced. After the TCE feed was introduced, a reduction in sMMO levels in the bioreactor were observed. Within 4 days the sMMO activity level in the bioreactor had fallen to 20 ± 1, a 73 percent decline. This significant decline in sMMO activity, as in the 10 mg/L TCE feed experiment, indicated a demonstrable TCE toxicity effect was occurring. Formate additions again commenced, with 0.2 M formate (pH 4.0) added at 0.1 mL/min. Formate caused only slow rise in sMMO activity levels which by 10 d had plateaued at 30 ± 2 nmol/h/mg cells, only a 50 percent improvement on the 4-day level and only 41 percent of the initial activity. TCE levels in the effluent slowly rose over the course of the experiment which by 13 days had reached 4.4 mg/L. In the experiment biomass also showed signs of a slow decline, at 4 days the biomass averaged 210 ± 20 mg/L, after 13 days this had fallen to 170 ± 20 mg/L.

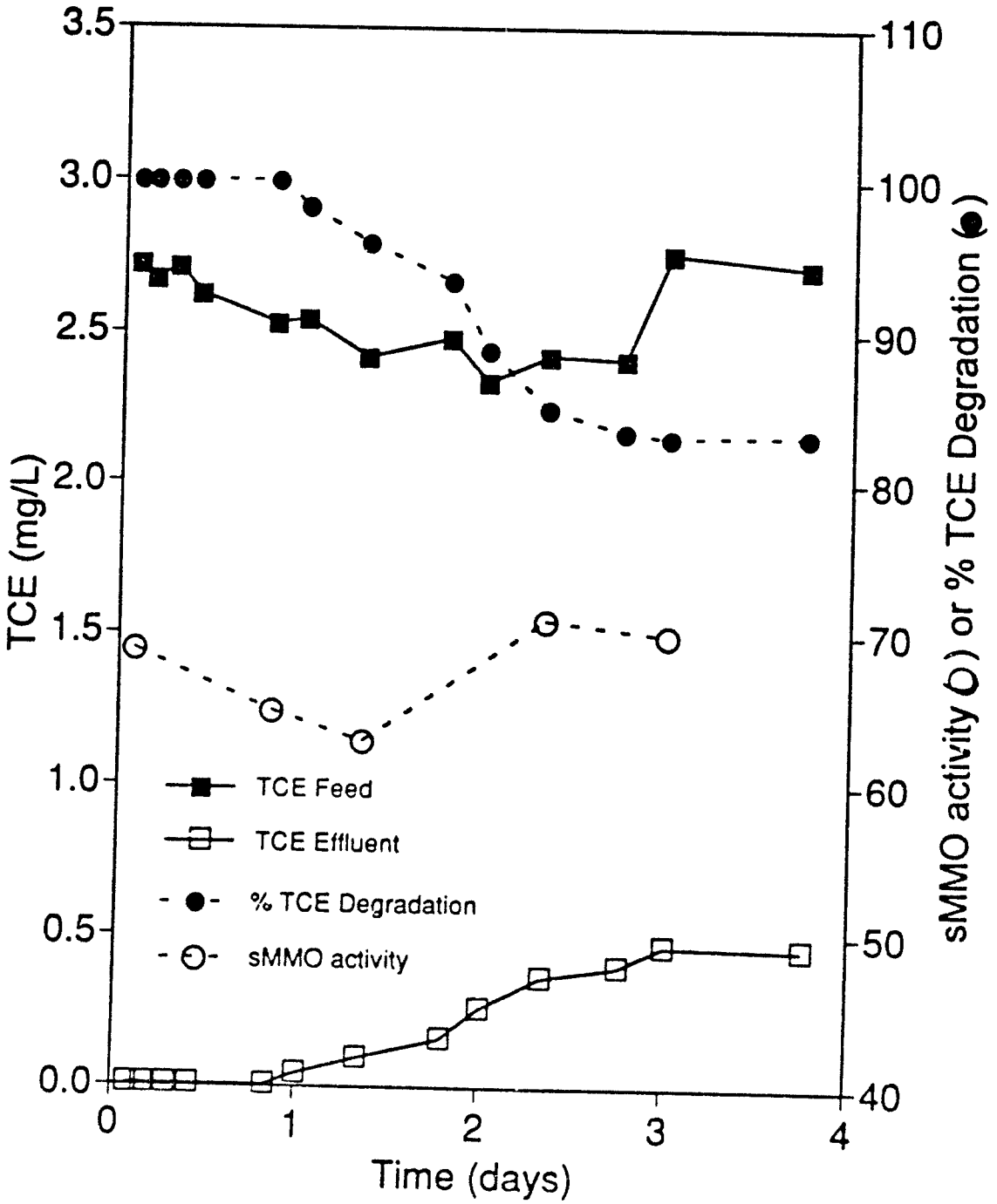

Figure 20. Single-pass experiment using a nominal TCE feed concentration of 3 mg/L.

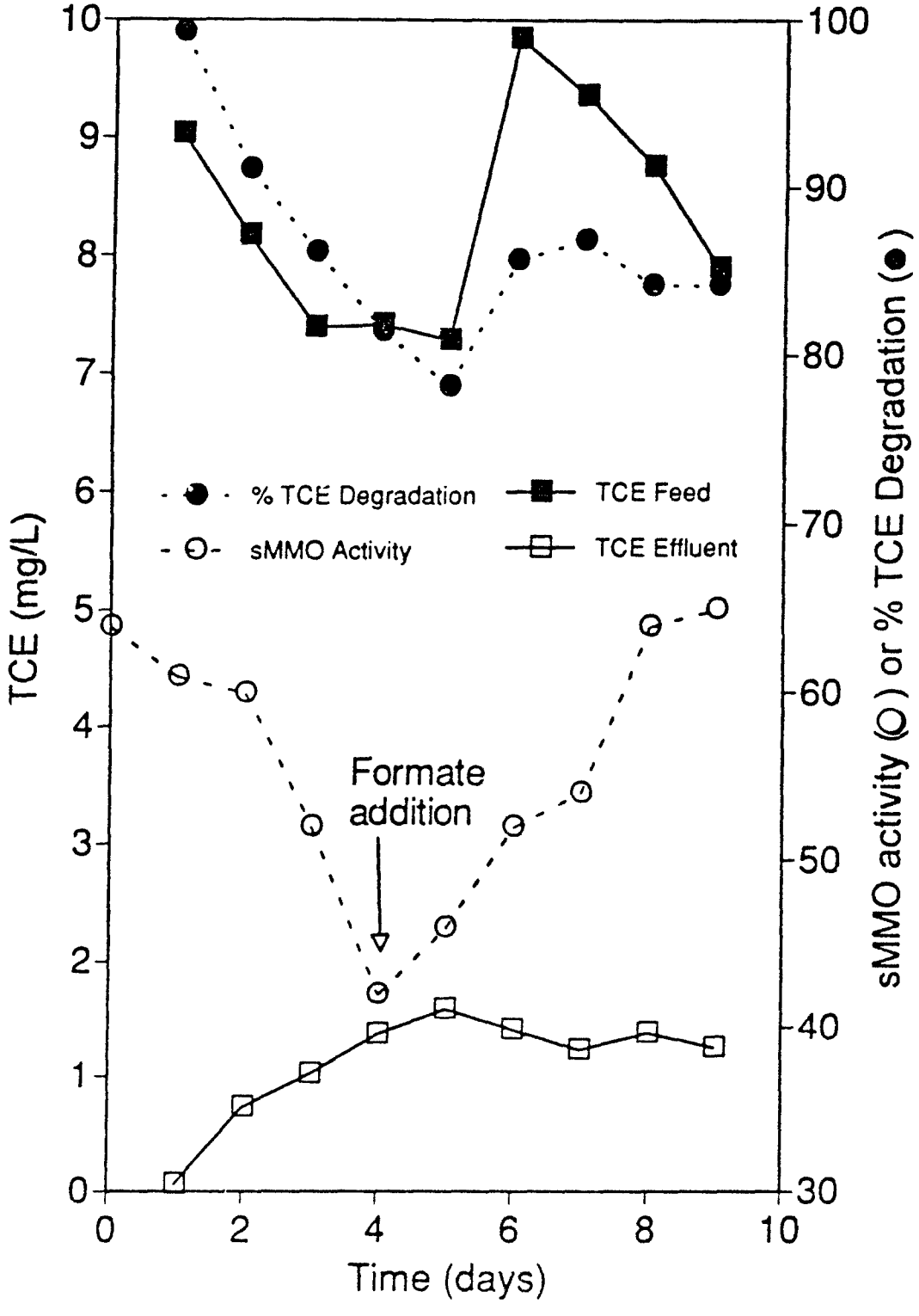

Figure 21. Single-pass experiment using a nominal TCE feed concentration of 10 mg/L. Continuous formate addition commenced after 4 days of operation.

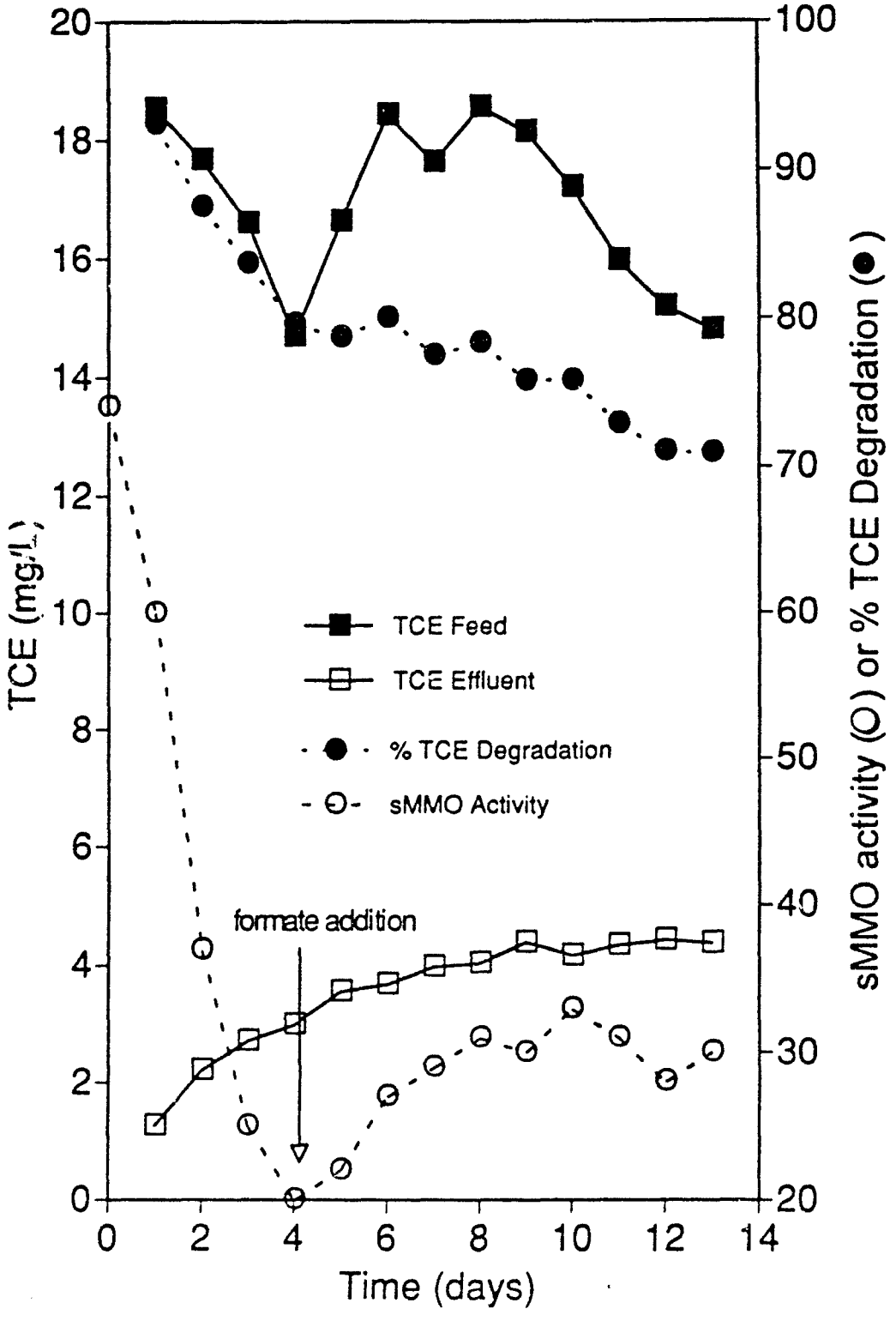

Figure 22. Single-pass experiment using a nominal TCE feed concentration of 20 mg/L.

4. Cross-flow TCE Degradation Experiments

a. 1 mg/L TCE Feed

In this experiment complete degradation of the 1 mg/L TCE feed was accomplished during the majority of the experiment which effectively lasted for 120 hours (5 days) (Figure 23). Unfortunately, this experiment ended prematurely because of combined problems associated with a sudden decrease in the temperature of the bioreactor (17° C) due to "unseasonable weather" and due to the failure of the dewatering device. Rapid washout of the biomass was the result. Feed levels for this experiment ranged from a low of 0.60 ± 0.08 mg/L to a high of 0.82 ± 0.07 mg/L. Even with low biomass levels TCE was completely removed by the fourth contacting column (C4). In this experiment biomass averaged only 110 ± 10 mg/, after 4 days. The sMMO activity of the cells in this experiment remained relatively stable ranging from 67 ± 6 to 80 ± 5 nmol/h/mg cells for the course of the experiment. No TCE toxicity effects were discernible.

b. 3 mg/L TCE Feed

In this experiment an average degradation of 91.3 percent ± 1.3 percent of the 3 mg/L TCE feed was accomplished with the experiment lasting 168 hours (7 days) (Figure 24). Feed levels for this experiment ranged from a low of 2.30 ± 0.07 mg/L to a high of 2.82 ± 0.15 mg/L. Effluent TCE levels averaged 0.22 ± 0.01 mg/L while the biomass stabilized at a value of 255 ± 15 mg/L between 4 and 7 days. The overall sMMO activity of the bioreactor biomass during this experiment showed a slight decline dropping from an initial value of 69 ± 4 down to 60 ± 2 nmol/h/mg cells. This decrease however did not suggest a statistically significant TCE toxicity effect.

c. 10 mg/L TCE Feed

In this experiment an average degradation of 93.7 percent ± 2.2 percent of the 10 mg/L TCE feed was accomplished with the experiment lasting 192 hours (8 days) (Figure 25). Feed levels for this experiment ranged from a low of 7.14 ± 1.13 mg/L to a high of 11.07 ± 0.11 mg/L. Effluent TCE levels averaged 0.45 ± 0.14 mg/L between 4 and 6 days while during this time the biomass averaged 395 ± 55 mg/L. The initial sMMO activity of the bioreactor averaged 77 ± 7 nmol/h/mg cells. Within 4 d of operation this value had fallen 70 percent to only 23 ± 6 nmol/h/mg cells. This significant decline in sMMO activity, as in previous single-pass experiments, indicated a demonstrable TCE toxicity effect was occurring. Formate additions were commenced, with 1.0 M sodium formate added at 0.1 mL/min, equivalent to an addition of 14.3 mM at the top of TCE contacting Column 3 following dilution. Formate caused a slow but significant rise in sMMO activity levels which by 7 days had reached 52 ± 13 nmol/h/mg cells, a 125 percent improvement on the 4-day level and 67 percent of the initial activity. Unfortunately, a rapid rise in bioreactor sample pH was also observed after 5 days, this resulted in significant decreases in biomass, sMMO activity and an increase in TCE levels by the last day of the experiment. After 8 days the pH had increased to 8.5 and was obviously becoming inhibitory to the biomass, thus the experiment was halted. Additionally formate levels had began to accumulate in the reactor ranging from 2-4 mM possibly resulting in additional inhibition to growth and sMMO activity. Changes in the formate introduction were implemented to avoid accumulation of formate and to maintain pH below 8.0 (see above for the single-pass 10 mg/L and 20 mg/L experiments).

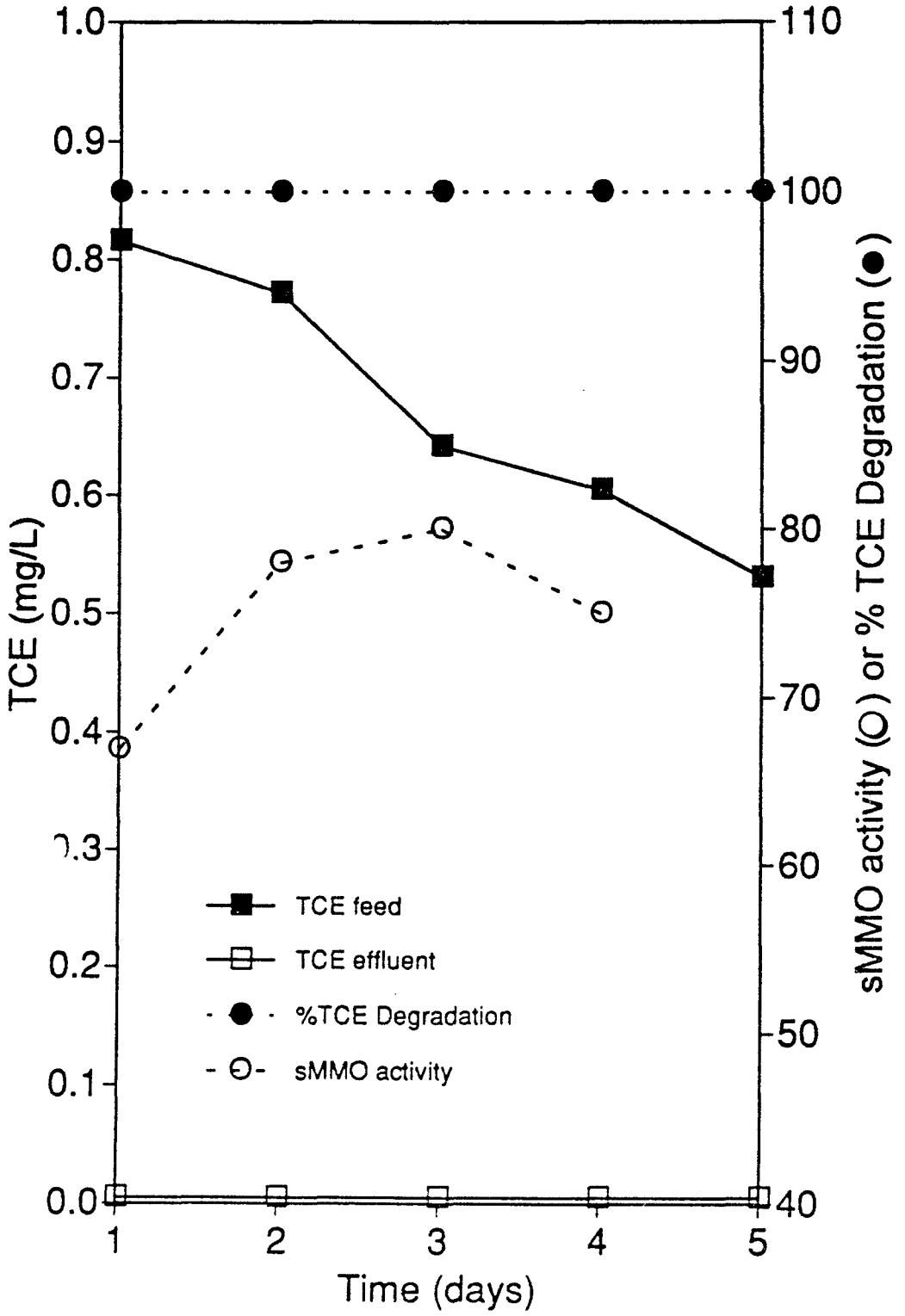

Figure 23. Cross-flow experiment using a nominal TCE feed concentration of 1.0 mg/L.

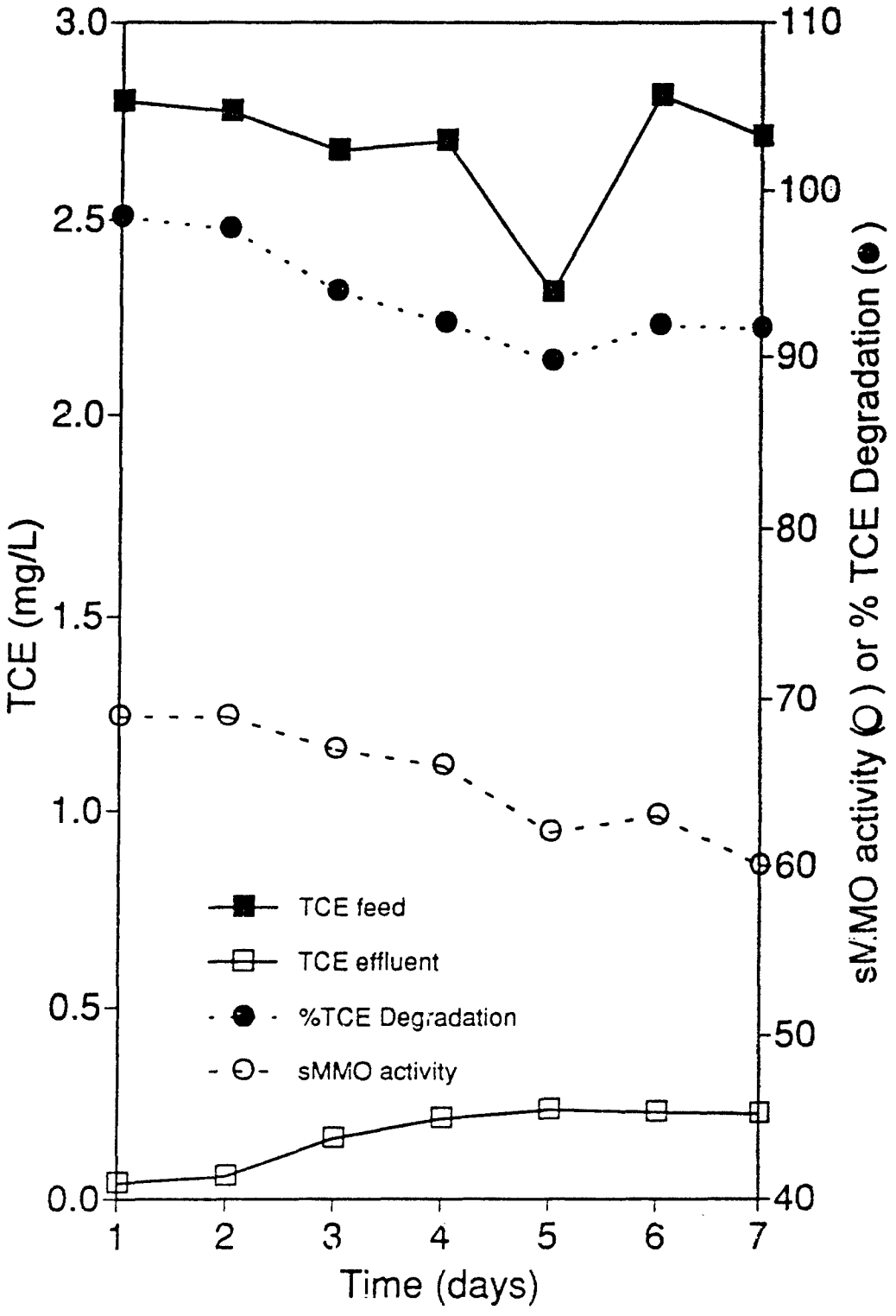

Figure 24. Cross-flow experiment using a nominal TCE feed concentration of 3 mg/L.

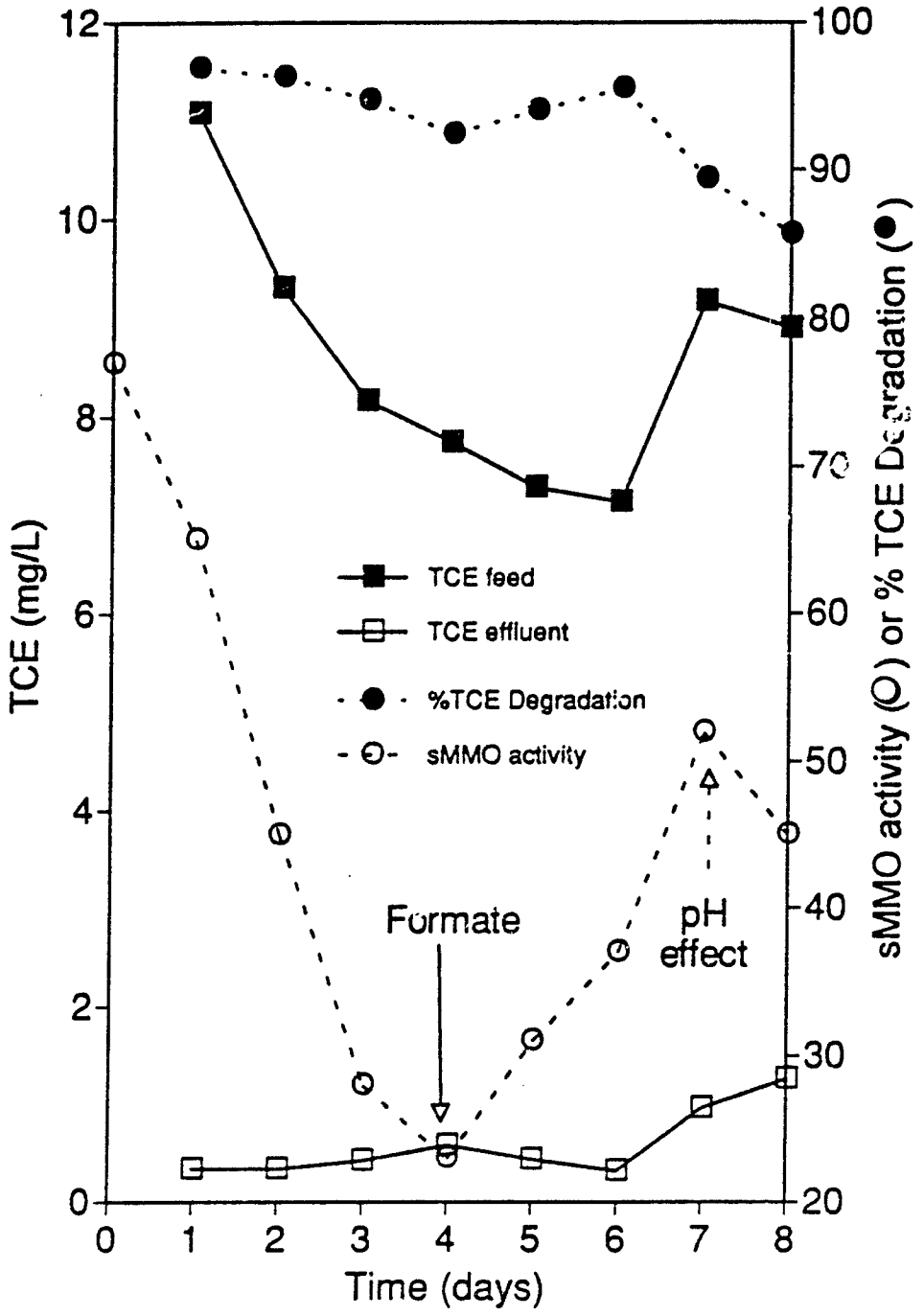

Figure 25. Cross-flow experiment using a nominal TCE feed concentration of 10 mg/L. Formate additions commenced after 4 days of operation. An unexpected pH increase began to cause inhibition after 7 days.

D. DISCUSSION

1. Continuous TCE Introduction

Abiotic experiments essentially showed the reactor did not lose TCE significantly through to biomass or bioreactor surfaces or lost through leakage or in the CSTR off-gas. Thus experimental results showed that the bioreactor successfully degraded a high proportion of inlet TCE concentrations ranging from 0.2 - 20 mg/L through biological degradation. Analysis of the bioreactor biomass indicated that TCE degradation was wholly due to the methanotroph *Methylosinus trichosporium* OB3b (PP358) and this is confirmable by the presence of sMMO activity at all times in the various experiments. The reactor degraded 0.2 mg/L TCE completely while operating in the single-pass mode while cross-flow mode appeared able to completely degrade 1 mg/L TCE. These levels of TCE are commonly found in polluted groundwater. The system seemed to be particularly stable in response to fluctuations in the TCE feed.

Experiments showed that the cross-flow mode seemed more efficient in removing TCE compared to the single pass. This is partly due to the slightly longer residence time of the cross-flow mode but also relates to whole cell TCE degradation kinetics. Figure 26 compares the average percent TCE degradation versus the average TCE effluent concentration obtained in the different experiments. Steady state was not obtained in the single-pass 20 mg/L TCE experiment in which TCE degradation exhibited a slow decay over time due to the high level of TCE toxicity (even with formate addition).

2. Optimization of sMMO

One of the advantages in using the constitutive mutant PP358 (Phelps et al., 1992; Fitch et al., 1993) is that copper suppression of sMMO was avoided. Copper suppression has been observed as a problem in other bioreactor studies (Fennell et al., 1993) reducing the rates of TCE degradation considerably. The only disadvantage in the use of PP358 is that its growth requirements are relatively fastidious. To obtain growth rates comparable to the wild-type OB3b, yeast extract and vitamins must be added to the NMS medium. Problems associated with overgrowth of the methanotroph by contaminants were not experienced though a significant (10 percent of the total), stable population of contaminants was present entering with the feed and colonizing the reactor. The contaminants had no effect on TCE. In order to obtain maximal levels of sMMO activity a variety of criteria were implemented. These criteria have been defined in batch culture optimization studies (Brusseau et al., 1990; Park et al., 1991; Section II). The most critical factor implemented was maintaining a high concentration of methane in the CSTR of the bioreactor. This allowed maximal growth of PP358, prevented exhaustion of reductant pools (i.e., NADH) needed for methane oxidation and TCE degradation, and helped recovery of sMMO activity if TCE induced any toxic effects on the cells or on sMMO. Methane competition is an important factor for TCE degradation. Fennell et al. (1993) showed in a methanotrophic attached-film expanded-bed (MAFEB) reactor that an increase of methane from 0.01 to 5.4 mg/L resulted in a 90 percent decline in TCE degradation rates. As most of the methane was consumed by the second contacting column competitive inhibition of TCE degradation levels were never observed. Brusseau et al. (1990) found that methane at 5-10 percent of solution saturation (1.6-3.2 mg/L methane) was optimal for TCE degradation by sMMO while Broholm et al. (1992) found that methane at levels less than 1.5 mg/L did not significantly affect TCE degradation. In the TCE contacting columns in which most of the TCE degradation takes place methane levels ranged from 0-0.9 mg/L.

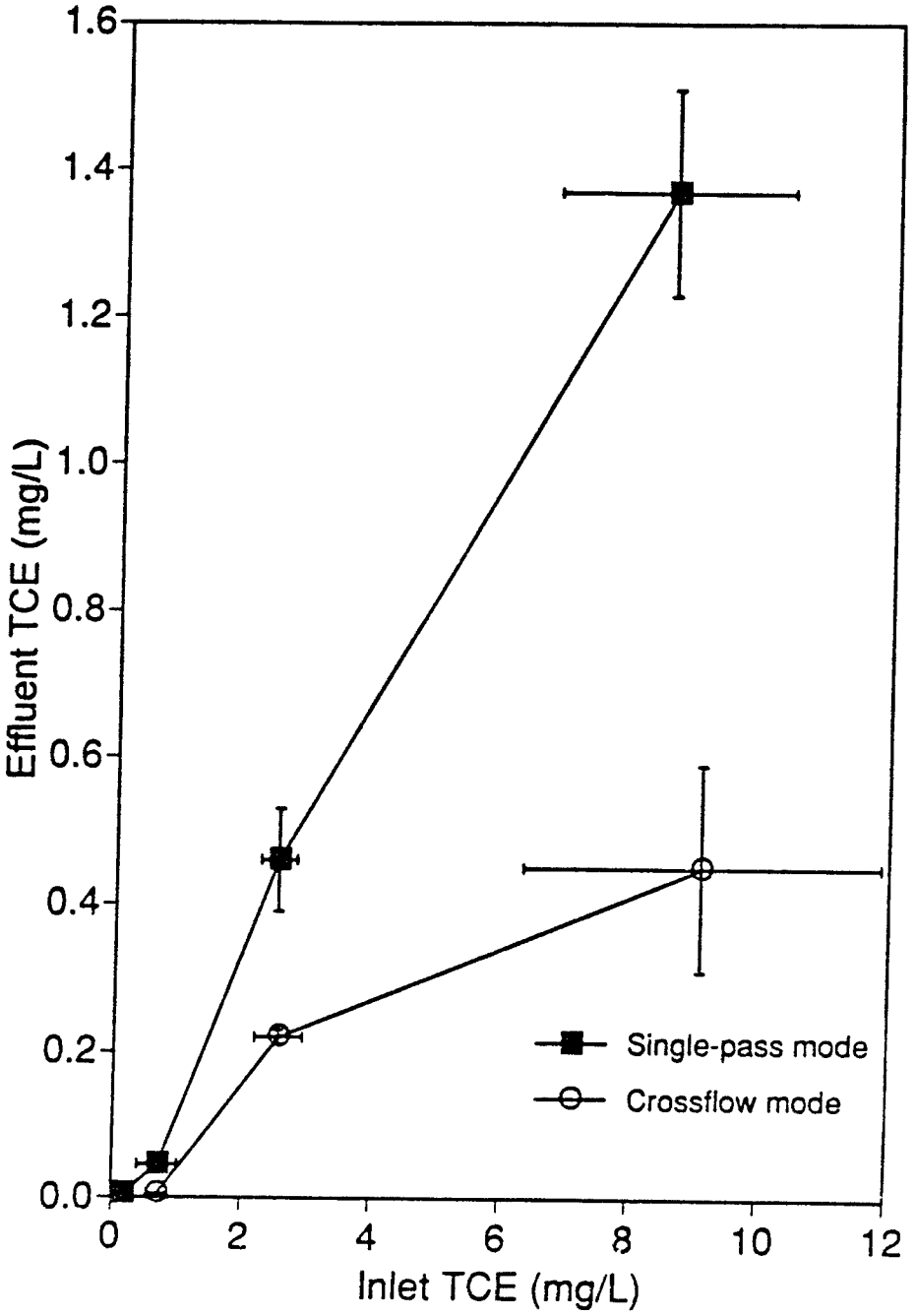

Figure 26. Comparison of the relative TCE degradation efficiencies of the single-pass and cross-flow bioreactor cell recycling modes.

A second factor implemented was maintenance of oxygen levels between 1 and 4 mg/L in the CSTR. This level was optimal for methanotroph growth and sMMO activity (Section II). When oxygen exceeds 5 mg/L lower growth rates and a reduction in sMMO activity occurs with subsequent biomass washout. In the bioreactor, during in all times of operation, oxygen was completely consumed by TCE contacting Column 4.

The feedstock maintained nitrate (2 mM), phosphate (2 mM), iron (50 μM), and magnesium (150 μM) levels well above limitation. Limitation of any of these nutrients leads to a decline in sMMO activity. During the various experiments these nutrients were always present in the bioreactor at levels adequate for high sMMO activity as compared to batch culture studies. The addition of a vitamin solution was needed to boost the growth rate of PP358 (Table 1).

3. sMMO Activity and TCE Toxicity Effects

It was interesting to observe that sMMO activity was relatively constant (50-70 nmol naphthol formed/hour/mg cell) in single-pass and cross-flow experiments using TCE feeds 3 mg/L or less (Figures 6-20, 23, and 24). The level of sMMO activity attained was approximately 25-30 percent of the maximal PP358 sMMO specific activity, as measured by the naphthalene oxidation assay using whole cells in the presence of 10 mM sodium formate (V_{max} = 200 nmol naphthol formed/hour/mg cells). Batch culture studies have shown sMMO specific activity tends to peak in early stationary growth phase then declines to a stable level. This is equivalent to what is observed in the bioreactor studied here. The stabilized sMMO activity could be maintained for several weeks in batch culture with periodical input of methane and nutrients (Section II).

Obvious toxicity effects were only observed after 4 days of operation in bioreactor experiments in which the TCE feed was 10 mg/L or greater (Figures 21, 22, and 25). Additionally, a more pronounced toxicity effect was so observable for the 10 mg/L TCE feed cross-flow experiment (a 70 percent inhibition) compared to the corresponding 10 mg/L TCE feed single-pass experiment (40 percent inhibition). This difference probably relates directly to the amount of TCE transformation taking place in the bioreactor. Since the biomass is staying at a relatively constant level, a greater degree of TCE transformation creates more TCE epoxide which inhibits sMMO. A greater degree of degradation of TCE was observable in the cross-flow experiments, compared to the single-pass experiments. The release of TCE epoxide was correspondingly greater, thus more toxicity was observed. The same effect is clearly observable in the 20 mg/L TCE feed single-pass experiment (no cross-flow experiment was performed with this TCE concentration). A 73 percent reduction was observed compared to 40 percent for the 10 mg/L single-pass experiment. The relative amount of degraded TCE almost doubled in the 20 mg/L TCE experiment leading to the much reduced sMMO levels. More experimentation is required to accurately determine the relationship between TCE degradation and sMMO activity inhibition in the bioreactor. A series of different TCE concentrations (starting at 5 mg/L) should be tested while trying to maintain all other system variables, i.e. biomass, oxygen and methane levels etc. relatively constant.

4. sMMO Recovery Using Formate Additions

The rationale for using formate to allow recovery and enhance TCE degradation has been presented in Section III of this report. When utilizing high TCE feed levels in certain bioreactor experiments, formate was added to combat a readily observable TCE toxicity effect. In two single-pass experiments performed using 10 mg/L and 20 mg/L TCE feed, and one cross-flow experiment

using 10 mg/L TCE feed formate was added after 4 d of operation. In the 10 mg/L operation formate enhanced TCE degradation and since biomass levels were relatively stable, formate also appeared to be aiding cell recovery from TCE toxicity (N.B. formate does not enhance growth rate, see Section II). In the single-pass 10 mg/L TCE feed experiments the activity level of sMMO returned to a point equivalent to the initial sMMO level. An even more pronounced recovery effect was observed in the 10 mg/L TCE feed cross-flow experiment (Figure 25). In these experiments, formate addition seemed to be successful in stabilizing the system and allowing a pseudo-steady-state to obtained. In the case of the 20 mg/L TCE feed single-pass experiment the formate caused a modest increase in sMMO levels which plateaued at only 40 percent of the initial activity (Figure 22). A significant decline in the relative amount of TCE being degraded continued to occur however, suggesting toxicity effects were still outstripping cell recovery rates. This could also be observed in the slowly declining biomass levels in the reactor towards the end of this experiment.

The only other research published using formate to enhance TCE degradation by methanotrophs was MacFarland et al. (1992). Using a dual-stage bioreactor, MacFarland et al. (1992) showed that the addition of formate (maintaining a steady state concentration of 20 mM) enhanced TCE degradation by a mixed methanotrophic consortia significantly. Overall, this study and these presented here indicate formate addition would be useful in enhancing TCE degradation rates, maintaining an active biomass, and stabilizing methanotroph-based biotreatment systems.

E. CONCLUSIONS

1. Abiotic experiments showed that the bioreactor was effective at maintaining a near complete mass balance for TCE degradation.

2. Experiments with TCE continuously introduced showed a high level of degradation with the relative amount of degradation declining with increasing TCE concentrations in the feed. Complete removal of TCE fed at 0.2 mg/L was achieved with the single-pass cell recycling mode while experiments indicated complete removal of TCE fed at 1 mg/L was possible with the cross-flow cell recycling mode.

3. The cross-flow mode of cell recycling appeared to be more efficient at removing TCE than the single-pass mode. Effluent levels were approximately 50 percent less in experiments using TCE inlet levels of 1, 3, and 10 mg/L.

4. TCE toxicity was observed if TCE feed concentrations were 10 mg/L or greater. The level of sMMO inhibition appeared to relate to the amount of TCE degradation however more experimentation is required to fully define this relationship.

5. The addition of formate was shown to enhance and stabilize reactor performance in the degradation of TCE fed at 10 mg/L with sMMO rising back to a level comparable to its original value.

SECTION V

MATHEMATICAL MODEL DEVELOPMENT

A. INTRODUCTION

The sampling ports located at the bottom of each of the plug-flow columns allow the extent of TCE degradation to be monitored throughout the series of columns, providing insight towards the kinetic behavior of the system. The CSTR was not considered during modelling, but only the plug-flow portion of the bioreactor was examined, due to the emphasis of biodegradation placed upon this unit (growth kinetics were not considered).

Alvarez-Cohen and McCarty (1991) have proposed a model for the treatment of halogenated aliphatic compounds in a two-stage dispersed growth unit, based on a second- order Monod equation:

$$r_s = \frac{kXS}{K_s + S} \tag{1}$$

where k is the maximum rate of contaminant transformation (d^{-1}), X is the active biomass (mg/L), S is the TCE concentration (mg/L), and K_s is the half-velocity constant for contaminant (mg/L).

To accommodate the toxicity of TCE towards the bacteria, including their sMMO supplies, a finite *transformation capacity*, T_c, was created, which is the maximum amount of TCE that may be degraded by a given quantity of cells (mg TCE/mg cells). Alvarez-Cohen and McCarty determined T_c to equal 0.0306 mg TCE/mg cell. This value was used in the model for this report.

B. MODEL IMPLEMENTATION

The transformation capacity is related to active microbial biomass by:

$$X = X_0 - \frac{1}{T_c}(S_0 - S) \tag{2}$$

where X_0 is the initial biomass concentration. The rate may be described as a differential, where:

$$\frac{dS}{dt} = -r_s \tag{3}$$

where t is time (days). In order to normalize the data to accommodate the cross-flow experiments' dilution characteristics in the plug-flow columns, the concentration of TCE, S, is related to a TCE mass flux, q, by:

$$q = S * F_T \tag{4}$$

where F_T is the total volumetric flow rate in the plug-flow columns. Combining Equations (1-4), a differential equation describing the decrease in TCE mass flux along the plug-flow reactor (expressed as volume of reactor, where V=0 is at the top of Column 1 and V=3.3 L is at the bottom of Column 4), is produced:

$$\frac{dq}{dV} = \frac{-k\left(X_0 - \frac{q_0 - q}{F_T T_c}\right)\frac{q}{F_T}}{K_s + \frac{q}{F_T}} \tag{5}$$

X_0 and F_T were determined experimentally. The half-velocity constant for TCE used was 16.56 mg/L (Bowman and Sayler, 1993).

Fluctuations in the TCE feed concentrations, due to partitioning of TCE between the liquid phase and the TCE feed storage tank headspace, and loss of biomass through the back-pressure relief valve out of the CSTR caused the reactor to behave in a non-steady state. However, the differential equation is intended for steady-state operation; therefore, regions where the system behaved in a pseudo-steady state was chosen as representative data for the system for each experiment. The pseudo-steady state was considered as the last three to five sampling points for each experiment, where the TCE concentrations were relatively stable throughout the reactor (Figures 27 to 31). The data points are joined by a spline curve to observe trends (DeltaGraph Professional, DeltaPoint, Inc., Monterey, CA). Also shown are the concentrations throughout the reactor for the cross-flow experiments (Figures 32 to 34).

Average biomass concentrations and flow rates were used for each experimental computation and is summarized in Table 7. The concentration of TCE (hence mass flux, q) at the top of Column 1 included a fraction from the TCE feed and a fraction from the recycled cell stream from the CSTR, which included undegraded TCE. Since this was an unmeasurable location in the reactor, q_0 was determined by performing a best-fit second order polynomial on the experimental data at the pseudo steady-state, and extrapolating the concentration at V=0 from the curve (see Figure 35). The results are also summarized in Table 7.

The model was fit to the data by adjusting k to match the final q generated by the model with that of q obtained experimentally at the bottom of Column 4 (V=3.297 Liters), and the results are shown in Figures 36 and 37. The obtained best-fit rate constants, shown in Figure 38, were averaged to obtain the average rate constant of $k_{ave} = 0.407\ d^{-1}$. Utilizing this average rate constant, the model

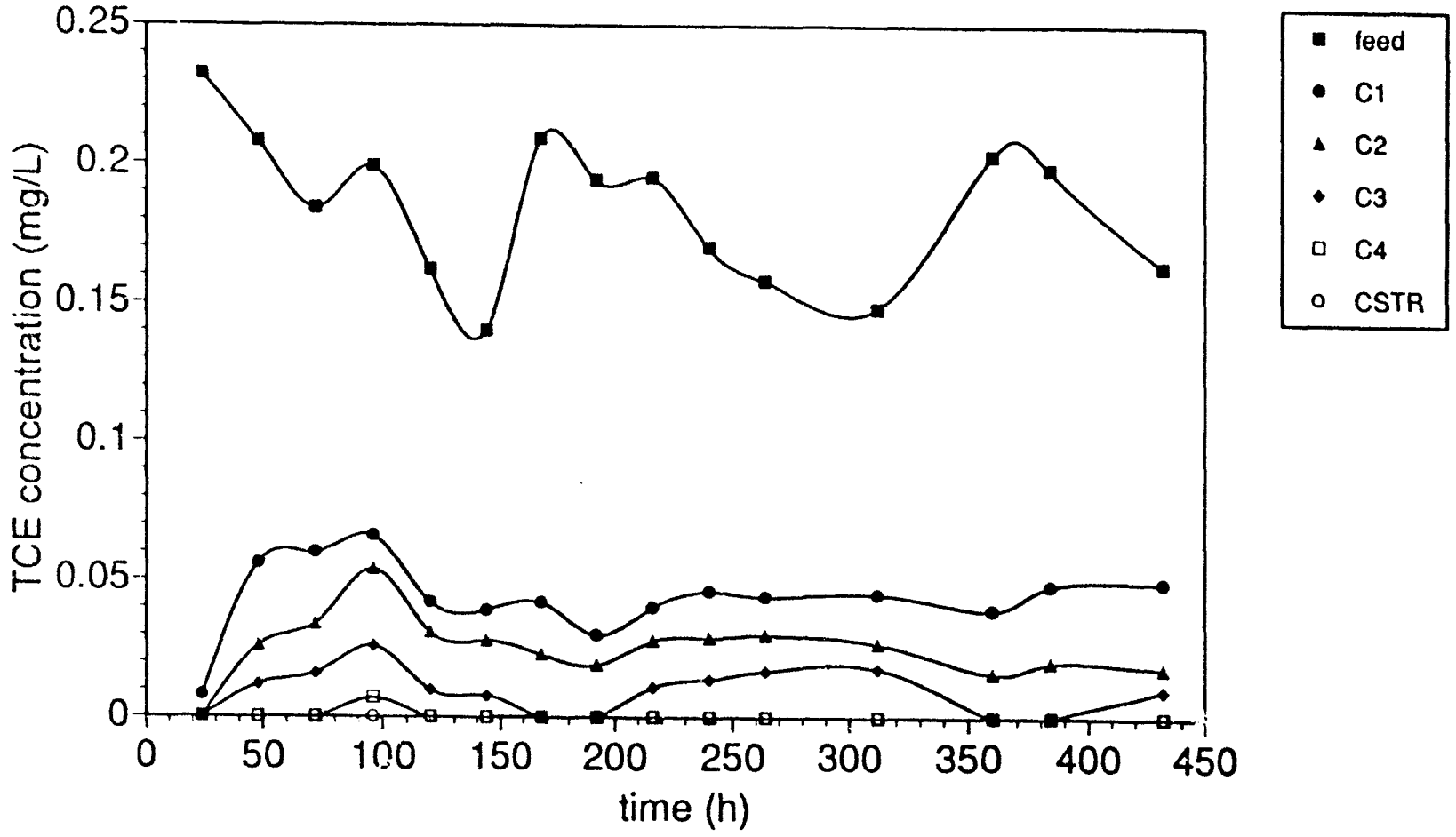

Figure 27. Bioreactor TCE concentration data for 0.2 mg/L nominal TCE feed concentration single-pass experiment.

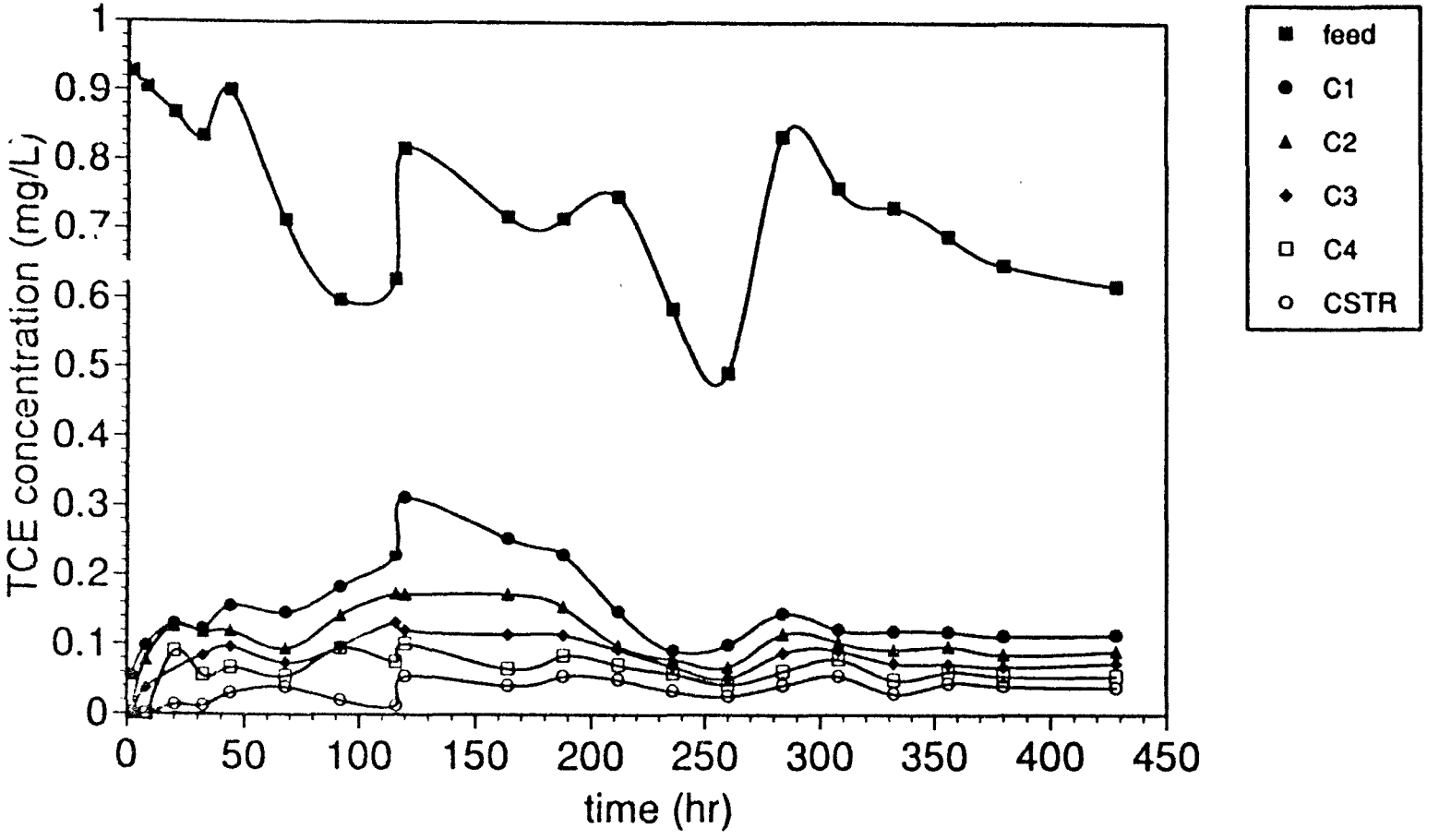

Figure 28. Bioreactor TCE concentration data for 1 mg/L nominal TCE feed concentration single-pass experiment.

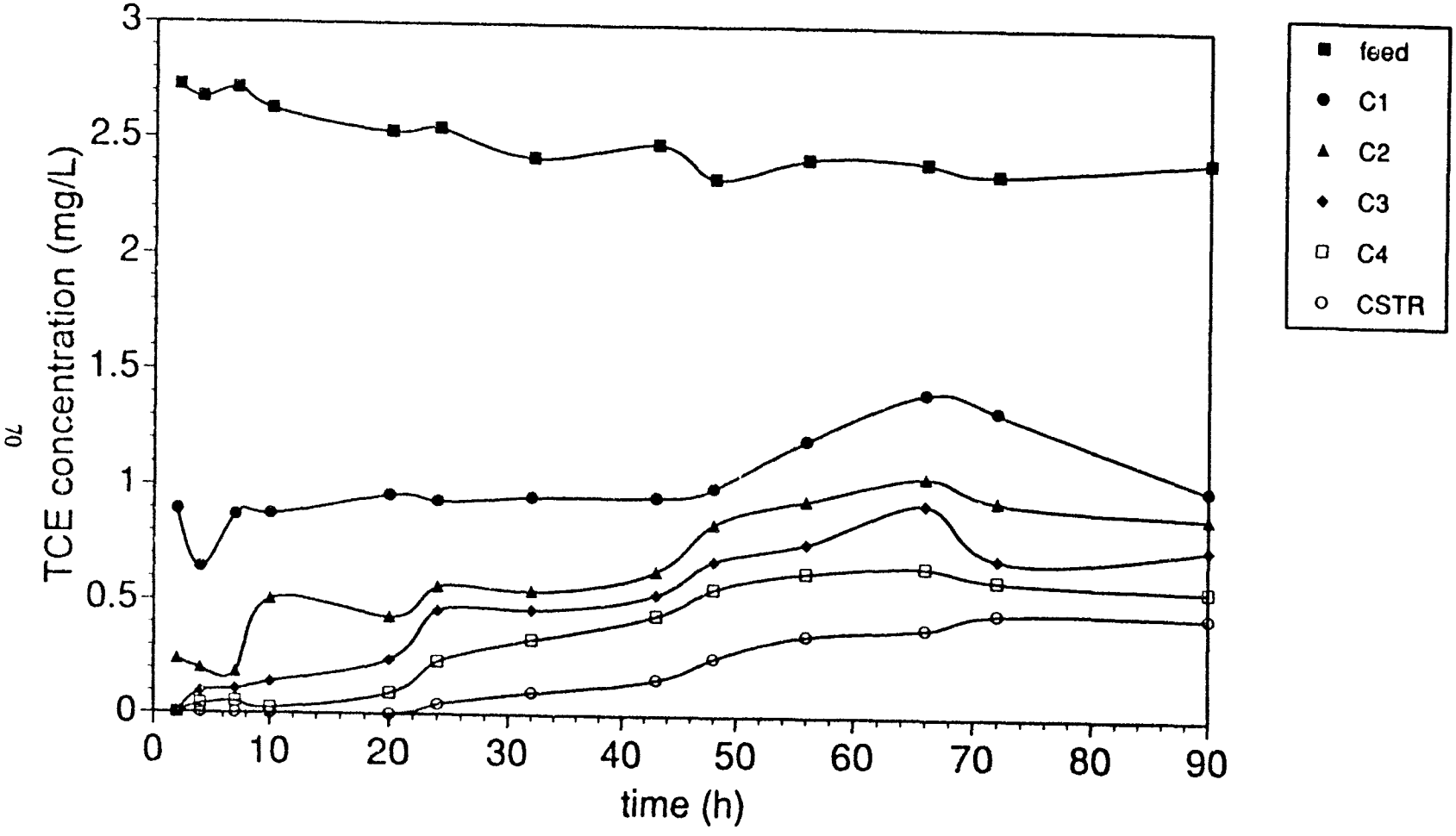

Figure 29. Bioreactor TCE concentration data for 3 mg/L nominal TCE feed concentration single-pass experiments.

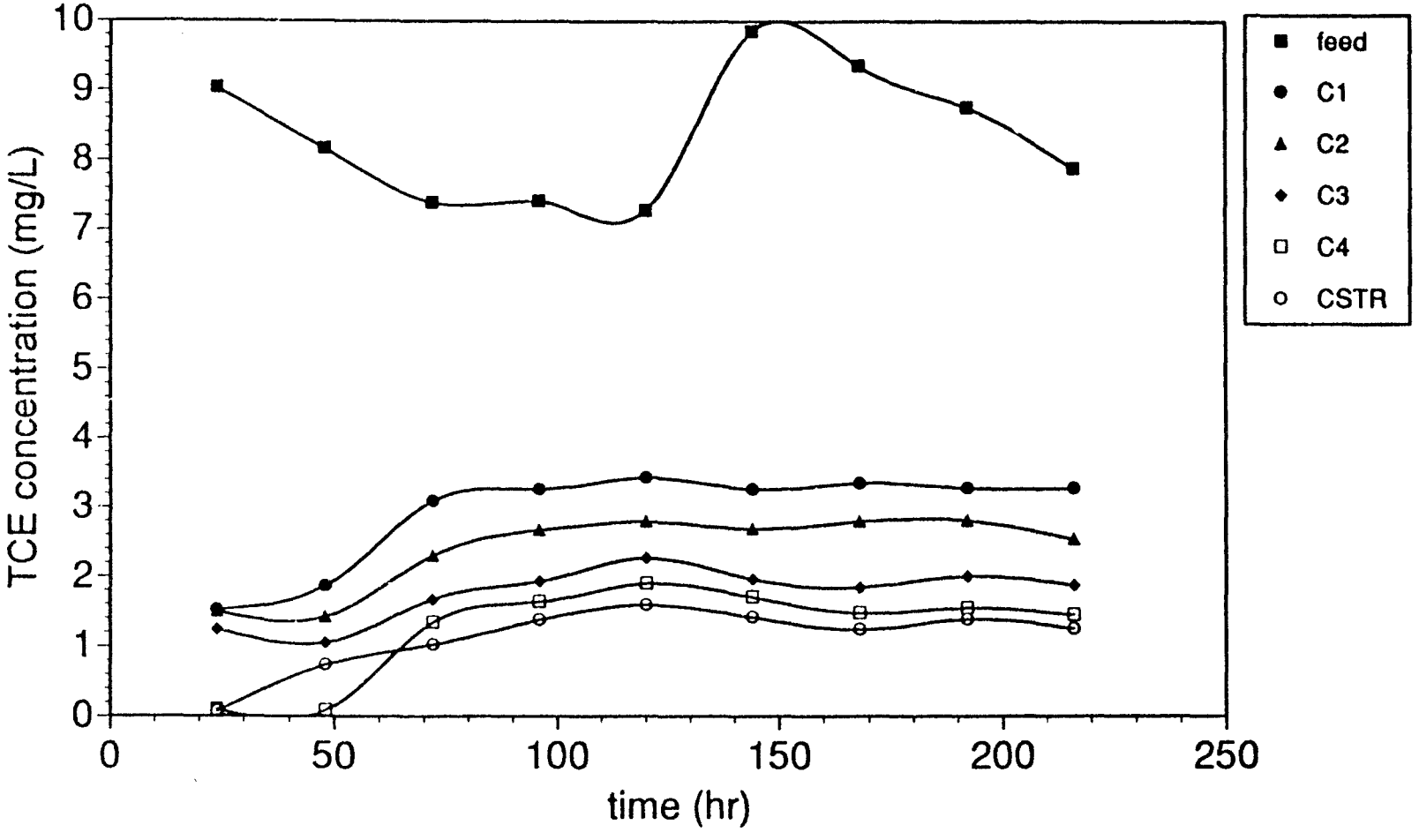

Figure 30. Bioreactor TCE concentration data for 10 mg/L nominal TCE feed concentration single-pass experiments.

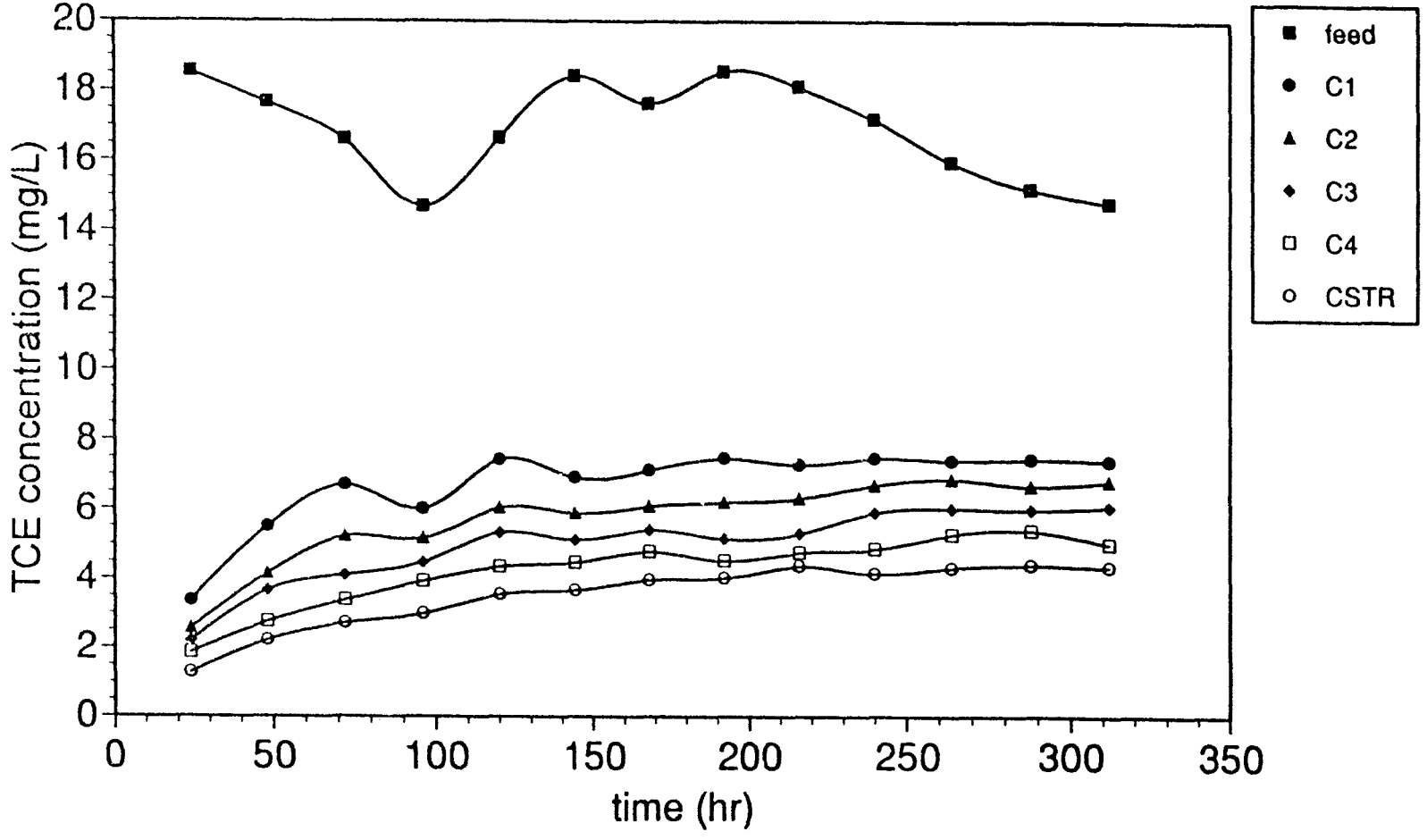

Figure 31. Bioreactor TCE concentration data for 20 mg/L nominal TCE feed concentration single-pass experiment.

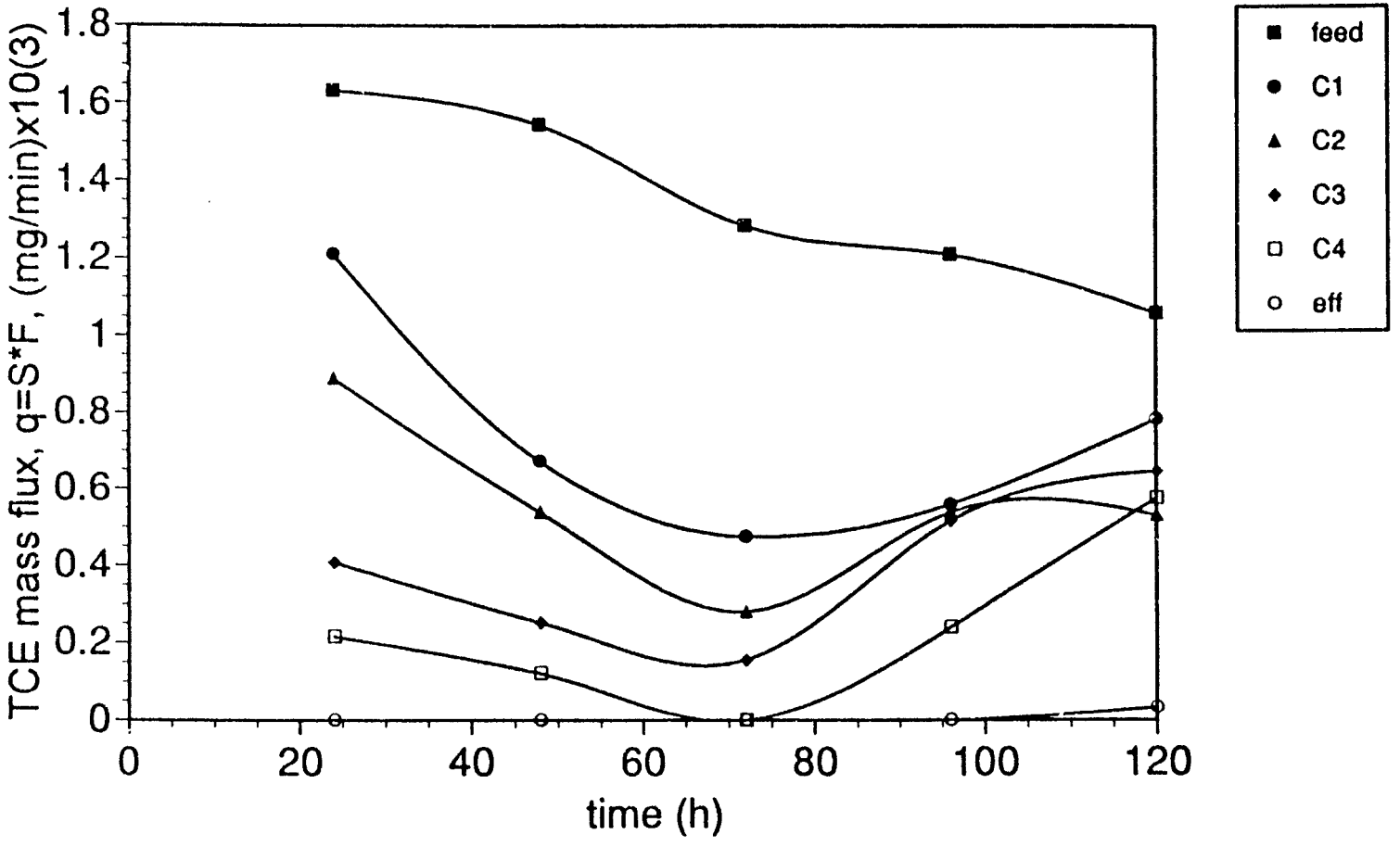

Figure 32. Bioreactor TCE concentration data for 1 mg/L nominal TCE feed concentration cross-flow experiment.

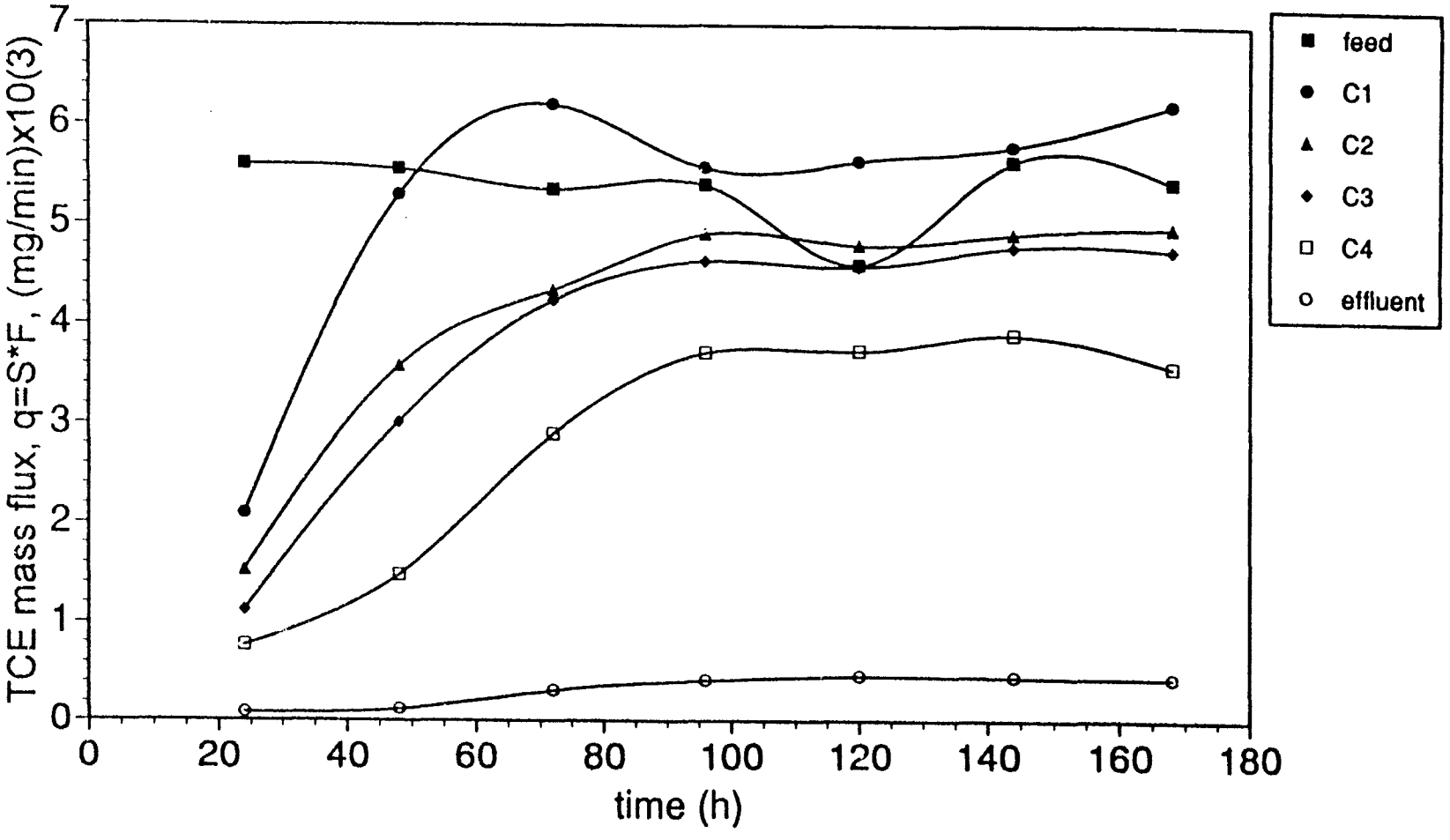

Figure 33. Bioreactor TCE concentration data for 3 mg/L nominal TCE feed concentration cross-flow experiment.

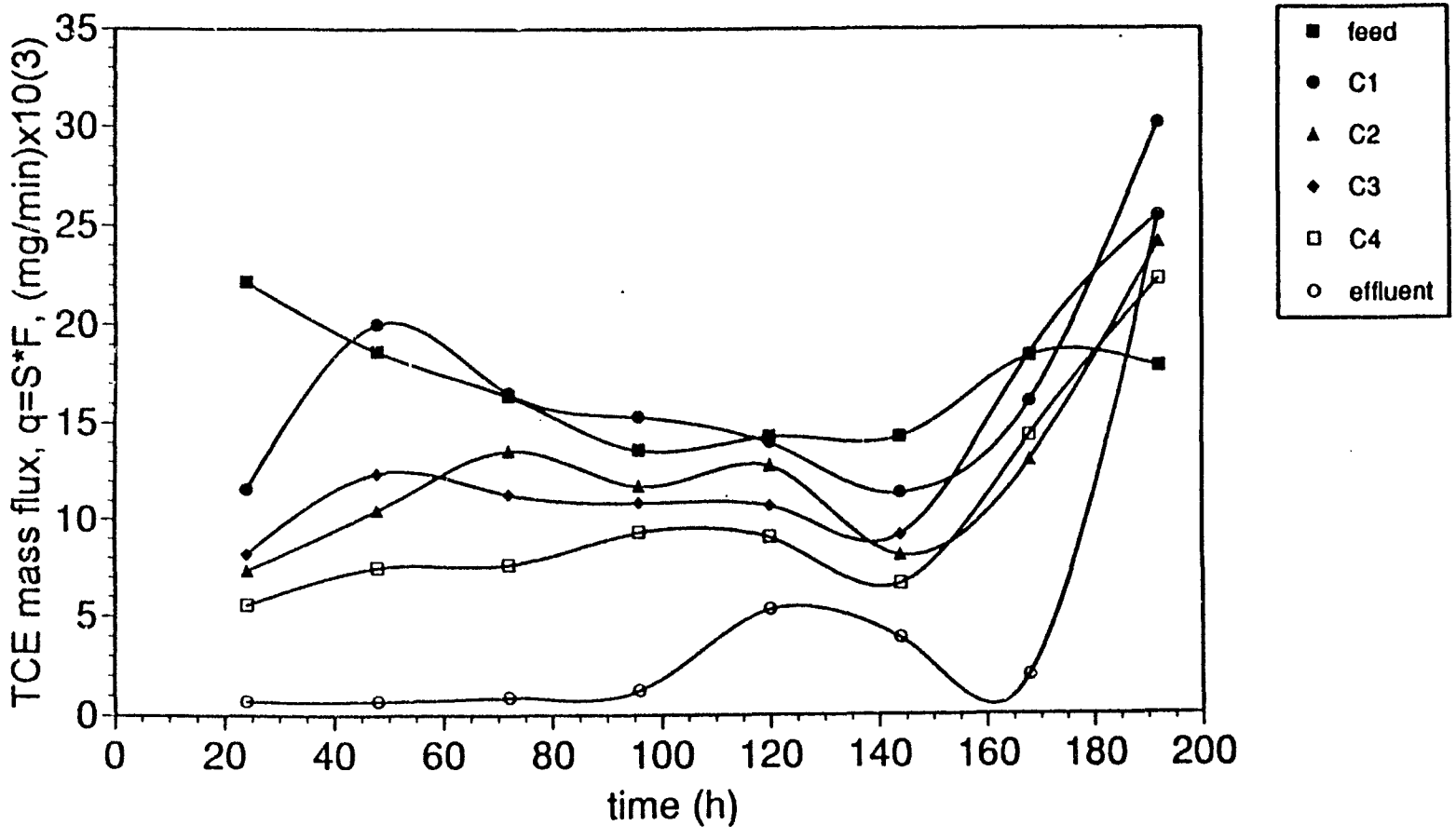

Figure 34. Bioreactor TCE concentration data for 10 mg/L nominal TCE feed concentration cross-flow experiment.

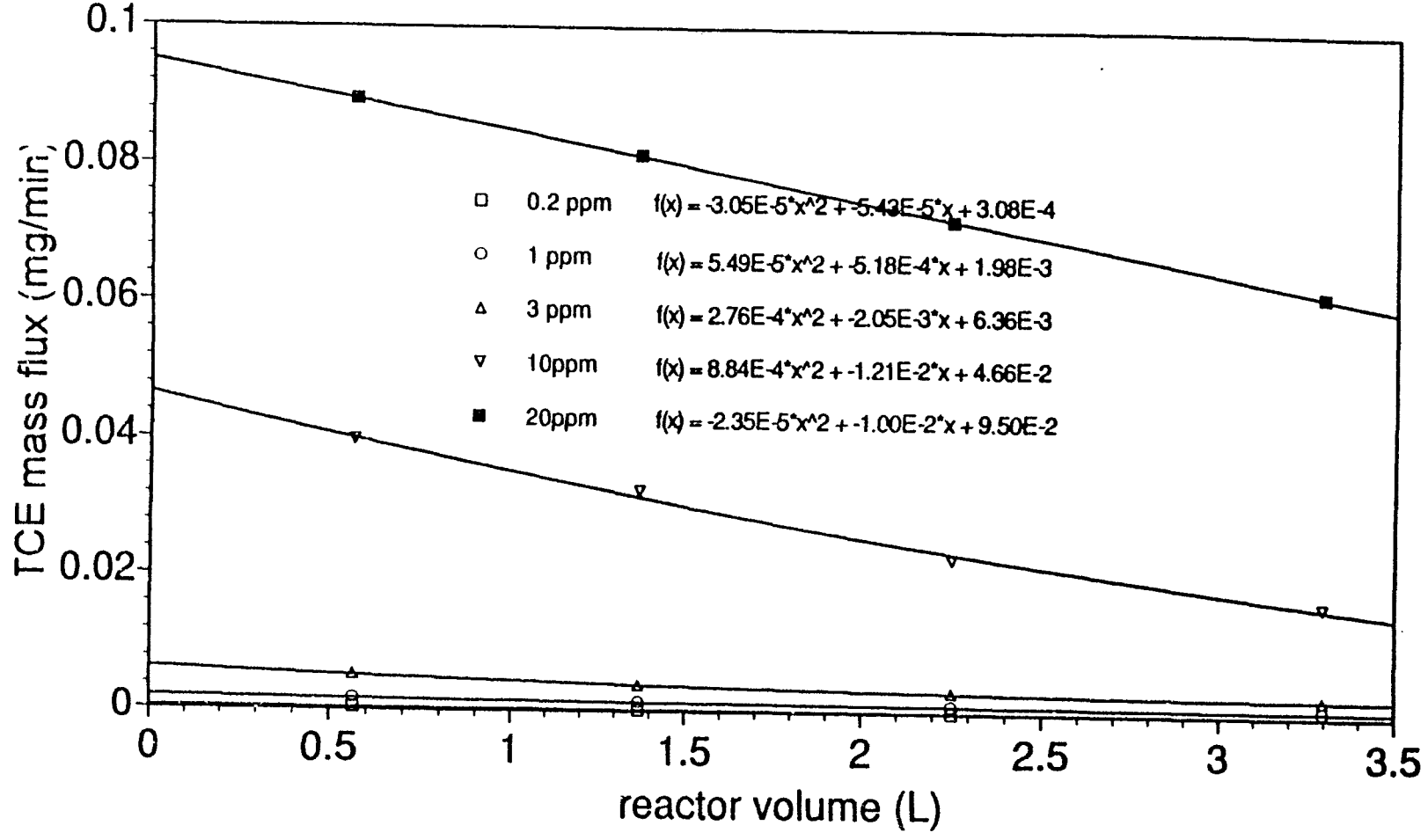

Figure 35. Fitting of data with second-order polynomial to obtain q0.

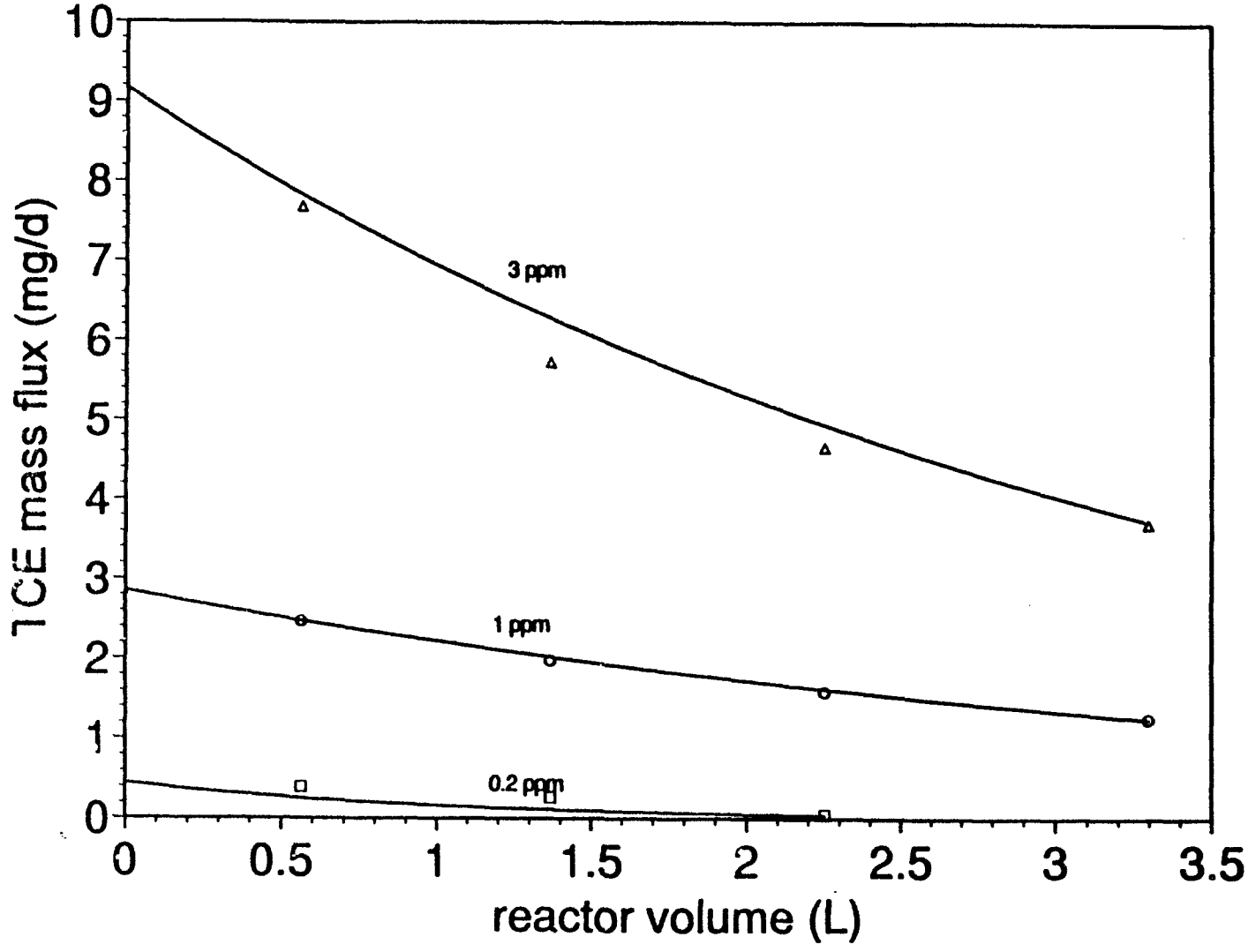

Figure 36. Best fit of McCarty model (solid lines) to 0.2 mg/L, 1 mg/L, and 3 mg/L nominal feed experimental data (symbols), based on matching final q of model with q at V=3.297 Liters of experimental data.

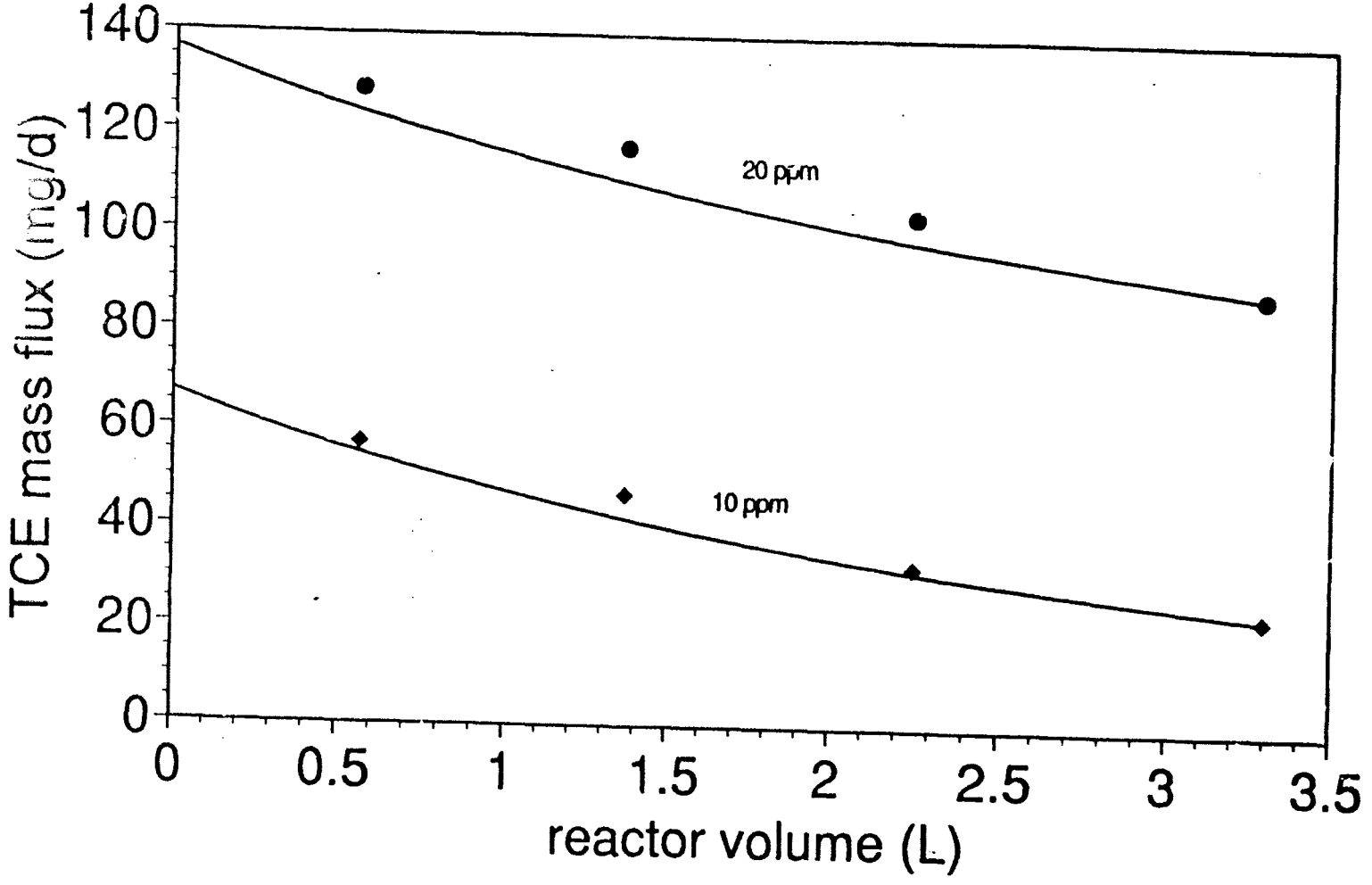

Figure 37. Best fit of McCarty model (solid lines) to 10 mg/L and 20 mg/L nominal feed experimental data (symbols), based on matching final q of model with q at V=3.297 Liters of experimental data.

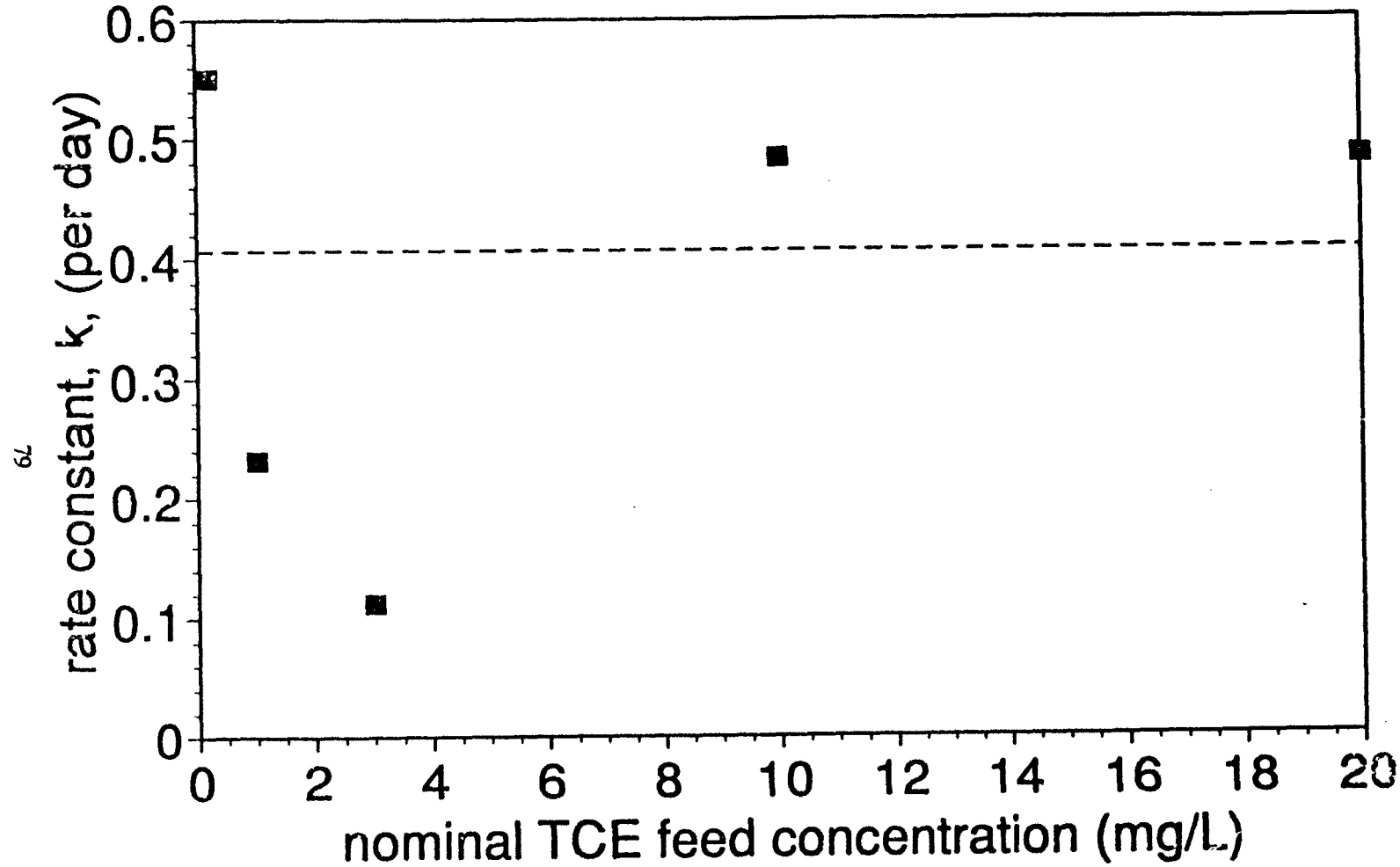

Figure 38. Best fit rate constants (symbols), k, for determination of the average rate constant (dashed line), kave.

TABLE 7. NOMINAL INFLUENT PARAMETERS AND CALCULATED TCE FLUX INTO CONTACTOR COLUMN 1 DURING OPERATION IN CROSS-FLOW MODE.

NOMINAL FEED CONCENTRATION	INITIAL BIOMASS CONCENTRATION	FLOW RATE	INITIAL TCE MASS FLUX
0.2 mg/L	310 mg/L	10 1 L/d	0.444 mg/d
1 mg/L	377 mg/L	20.9 L/d	2.85 mg/d
3 mg/L	315 mg/L	7.07 L/d	9.16 mg/d
10 mg/L	266 mg/L	17.3 L/d	67.10 mg/d
20 mg/L	161 mg/L	17.3 L/d	136.8 mg/d

was again compared to the data, and showed good agreement (as shown in Figure 39), except in the case of the 3 mg/L experiment. This could be partly due to not running the 3 mg/L experiment for a long enough period of time, resulting in the system not having adequate time to reach a pseudo-steady state. All other single pass experiments were run for over 200 hours and had adequate time to reach the pseudo-steady-state.

In order to test the strength of the toxicity term in the Alvarez-Cohen and McCarty model, the data were also compared to the standard second-order model:

$$\frac{dS}{dt} = \frac{-kXS}{K_s + S} \tag{6}$$

adjusted to mass flux:

$$\frac{dq}{dV} = \frac{-kX_0 \frac{S}{F_T}}{K_s + \frac{S}{F_T}} \tag{7}$$

The model was fitted to the 0.2 mg/L, 1 mg/L, and 3 mg/L nominal concentration experiments by adjusting the final q to the values of q experimentally determined at the bottom of Column 4 and is shown in Figure 40. The experimental data matched the model very well for the 0.2 mg/L and the 1 mg/L experiment, but again showed deviation for the 3 mg/L experiment. However, it was also found that the same rate constant was determined for the best-fit procedure for both models. In both cases, $k = 0.55\ day^{-1}$ for the 0.2 mg/L case, and $k = 0.231\ day^{-1}$ for the 1 mg/L case. This demonstrates, that at low concentrations, the toxicity term in McCarty's model has little impact on the rate of degradation, due to the small value of S.

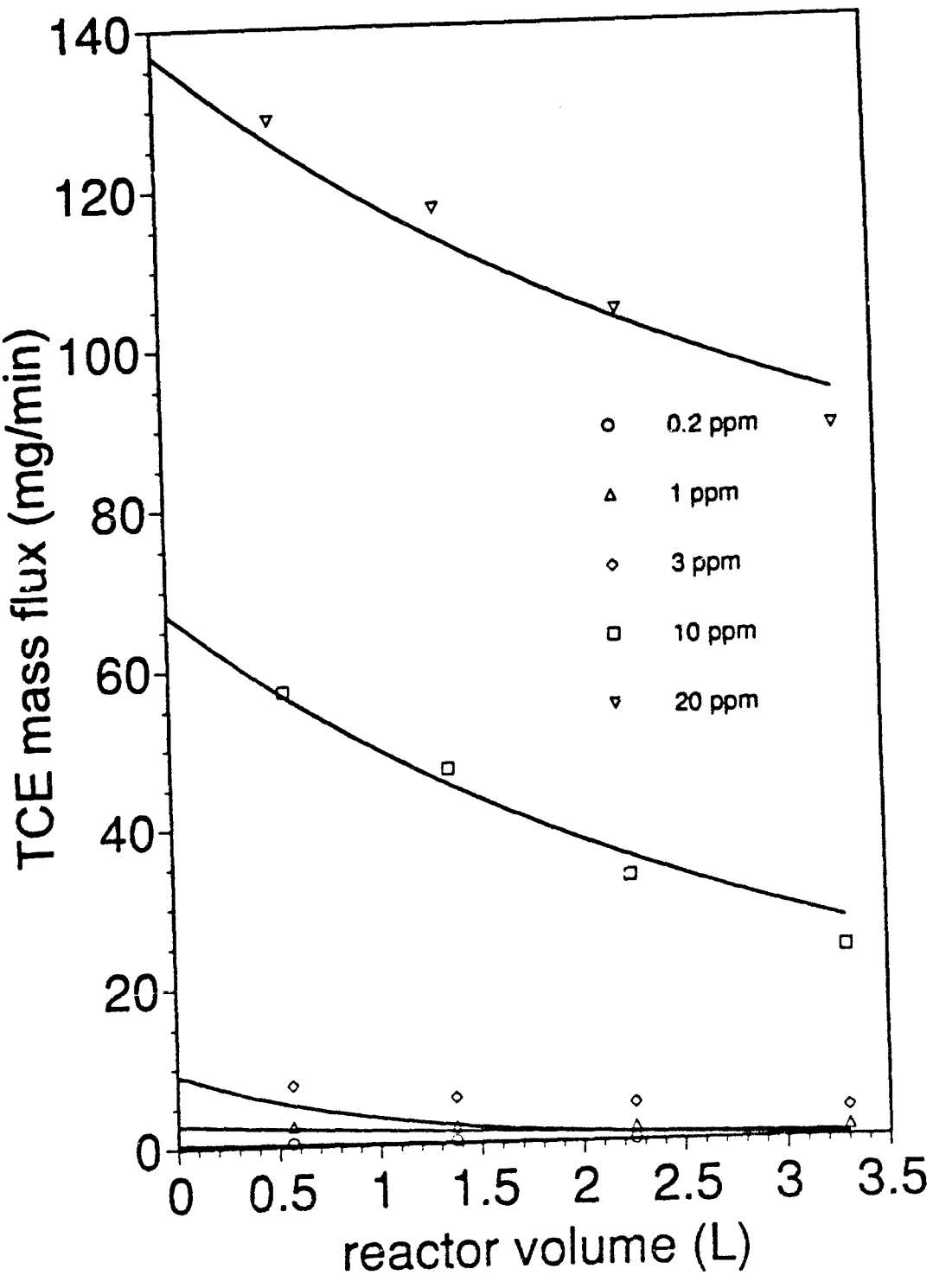

Figure 39. Comparison of experimental data (symbols) with model (solid lines) utilizing k_{ave}.

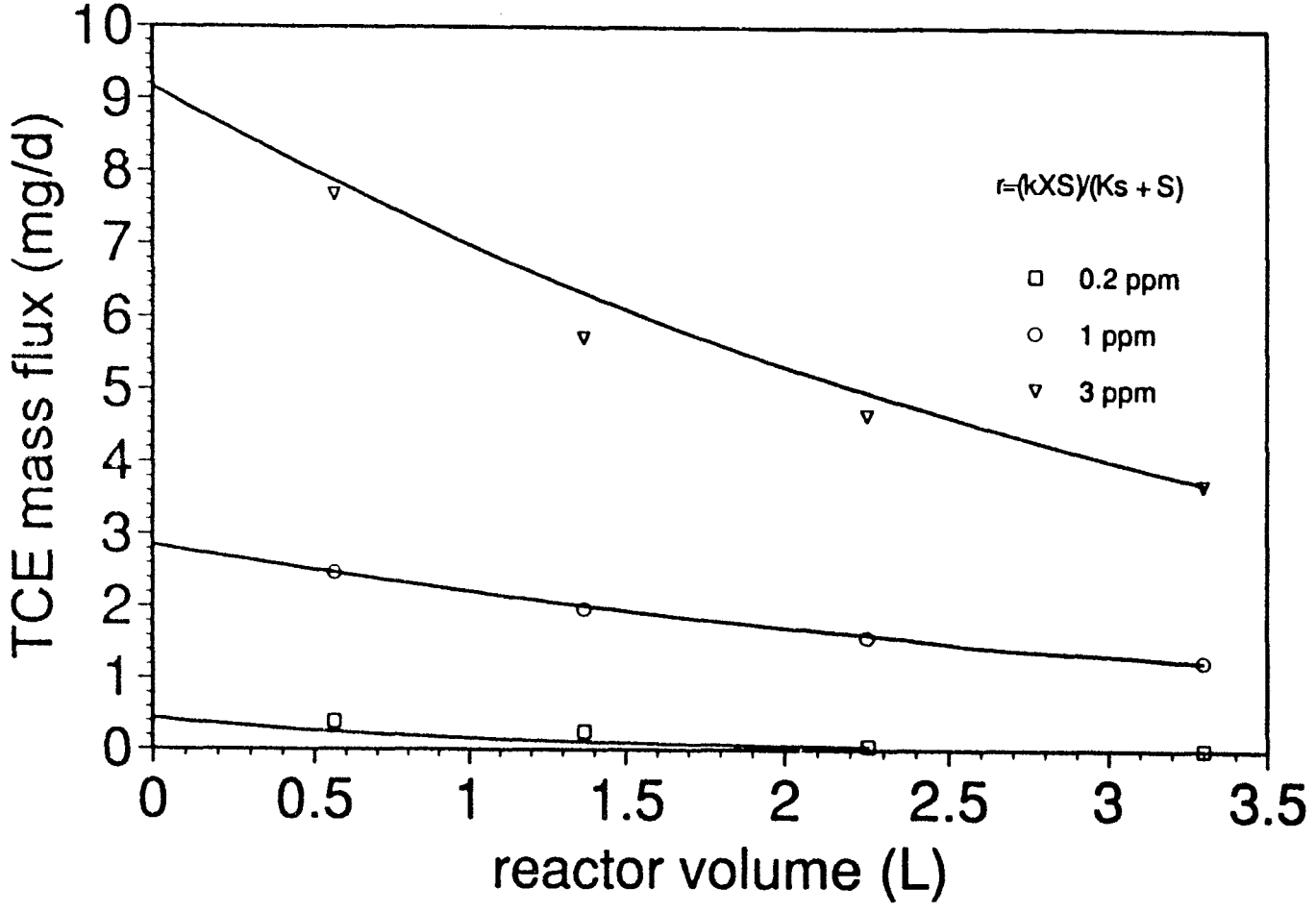

Figure 40. Best fit of simple second-order model (solid lines) with 0.2 mg/L, 1 mg/L, and 3 mg/L nominal feed experimental data (symbols), based on matching final q of model with q at V=3.297 Liters of experimental data.

Since the Alvarez-Cohen and McCarty's toxicity term only has an effect on a large value of S, one should see a better fit of the McCarty model to the higher concentration experiments of 10 mg/L and 20 mg/L than the simple second-order equation, if toxicity were indeed occurring. Therefore, the best-fit rates of the 0.2 mg/L and 1 mg/L experiments were averaged (obtaining k_{ave}= 0.39 day^{-1}) and used to compare the two models against the 10 mg/L and 20 mg/L data. The results are shown in Figure 41. At 10 mg/L, it appears that the simple second-order model represents the data more closely than Alvarez-Cohen and McCarty's model, but at 20 mg/L, the toxicity term drives the model prediction towards the data. Therefore, one does not see an advantage in Alvarez-Cohen and McCarty's toxicity term, until the 20 mg/L experiment, where the mass flux through the reactor is as high as 137 mg/d. At concentrations lower than this, the simple model predicts the data as well, if not better, than the Alvarez-Cohen and McCarty model.

Applying a best-fit of the model to the data to obtain the rate constants makes the models empirical, even though they are backed by a theoretical argument. This is especially true for the Alvarez-Cohen and McCarty model, where the biomass was logically diminished over time, by contact with TCE, which has theoretical, as well as experimental backing. However, the models are based on maximum rates, and in the bioreactor, where the conditions are less than optimal for maximum degradation (i.e. contaminant bacteria, less than optimal sMMO activity, etc.), it is difficult to correlate the rate of degradation with biomass levels. The equation should include terms for sMMO levels, rather than biomass, in order to make the model "theoretical" rather than empirical.

If the models were assumed accurate over the range of TCE feed concentrations, then the models may be used to compare the single-pass mode with the cross-flow mode of operation of the bioreactor. The experimental data suggested that the cross-flow mode was more efficient in the degradation of TCE (see Section IV), where essentially 100 percent of 1 mg/L (nominal concentration) influent TCE was removed during cross-flow operation as opposed to 95 percent for single-pass operation. Furthermore, during the 10 mg/L experiments, 94 percent of the influent TCE was removed during cross-flow operation, as opposed to 83 percent for the single-pass operation. However, differences in biomass concentrations and actual influent TCE concentrations made comparison of the two modes of operation difficult. Since the model has been shown to represent the data over the experimental ranges, the Alvarez-Cohen and McCarty model was used to compare the two modes.

To use the Alvarez-Cohen and McCarty model to compare the two modes of operation in the plug-flow portion of the bioreactor, several assumptions were made:

(1) No degradation of TCE occurs in the CSTR, so that the recycle streams from the CSTR to the plug-flow Columns are of the same TCE concentration as the effluent of Column 4.
(2) Cell deactivation in the plug-flow columns is cumulative.
(3) The cells are completely reactivated in the CSTR.

The parameters used in the model are: k = 0.39 day^{-1}, the biomass concentration in the CSTR is 360 mg/L, F_{rec}=0.010 L/min (to Column 1) for the single-pass mode, F_{rec}=0.005 L/min (to Columns 1 and 3) for the cross-flow mode, and F_{feed}=0.002 L/min. The two modes were compared for nominal TCE feed concentrations of 1 mg/L, 10 mg/L, and 20 mg/L, or influent mass flux rates, q_{feed}, of 2.88

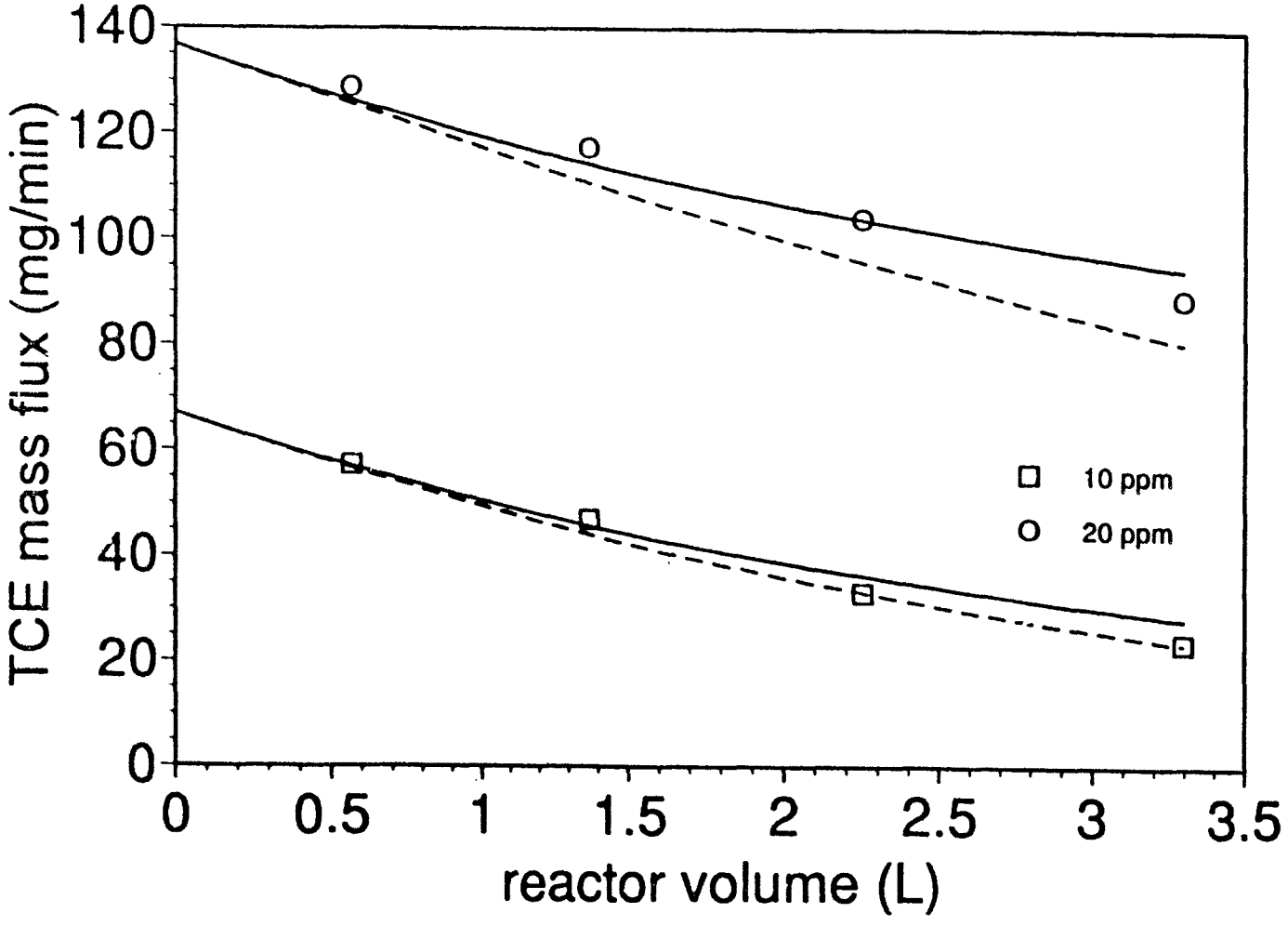

Figure 41. Comparison of McCarty model (solid lines) with simple second-order model (dashed lines) to 10 mg/L and 20 mg/L nominal feed experimental data (symbols).

mg/d, 28.8 mg/d, and 57.6 mg/d, respectively. The TCE mass flux at the top of Column 1, q_0 (mg/d), may be written as the sum:

$$q_{rec} + q_{feed} = q_0 \tag{8}$$

where q_{rec} is the TCE mass flux of the recycle stream from the CSTR to the top of Column 1 (mg/d). A dilution equation was used to calculate the biomass at the top of Column 1, X_0, with a feed rate of 2mL/min and a biomass concentration in the CSTR of 360 mg/L:

$$X_0 = \frac{F_{rec}}{F_{rec} + 2mL/\min} 360 mg cells/L \tag{9}$$

where F_{rec} is the flow rate of the recycle stream from the CSTR to the top of Column 1. The model was run for the three concentrations in the single-pass mode by iterating the model and matching q_{rec} with a diluted TCE mass flux exiting Column 4 (q_e):

$$q_{rec} = \frac{F_{rec}}{F_T} q_e \tag{10}$$

where F_T is the total flow rate exiting Column 4 (L/day). The results are shown in Figure 42.

The model was then adjusted to accommodate the cross-flow mode of operation, by running the model first over the volume of the first two columns (upper units, designated by subscript u), then again over the last two columns (lower units, designated by subscript l). The recycle flow rates from the CSTR to the top of Column 1 and the top of Column 3, each equalled $F_{rec\_single\_pass}/2$ = 0.005 L/min. The q_0 for the second of the runs ($q_{0,l}$ located at the top of Column 3) is the sum of:

$$q_{e,u} + q_{rec} = q_{0,l} \tag{11}$$

where $q_{e,u}$ is the final TCE mass flux at the bottom of Column 2 (computed from first run of model), and q_{rec} is the TCE mass flux from the recycle stream from the CSTR to Column 3.

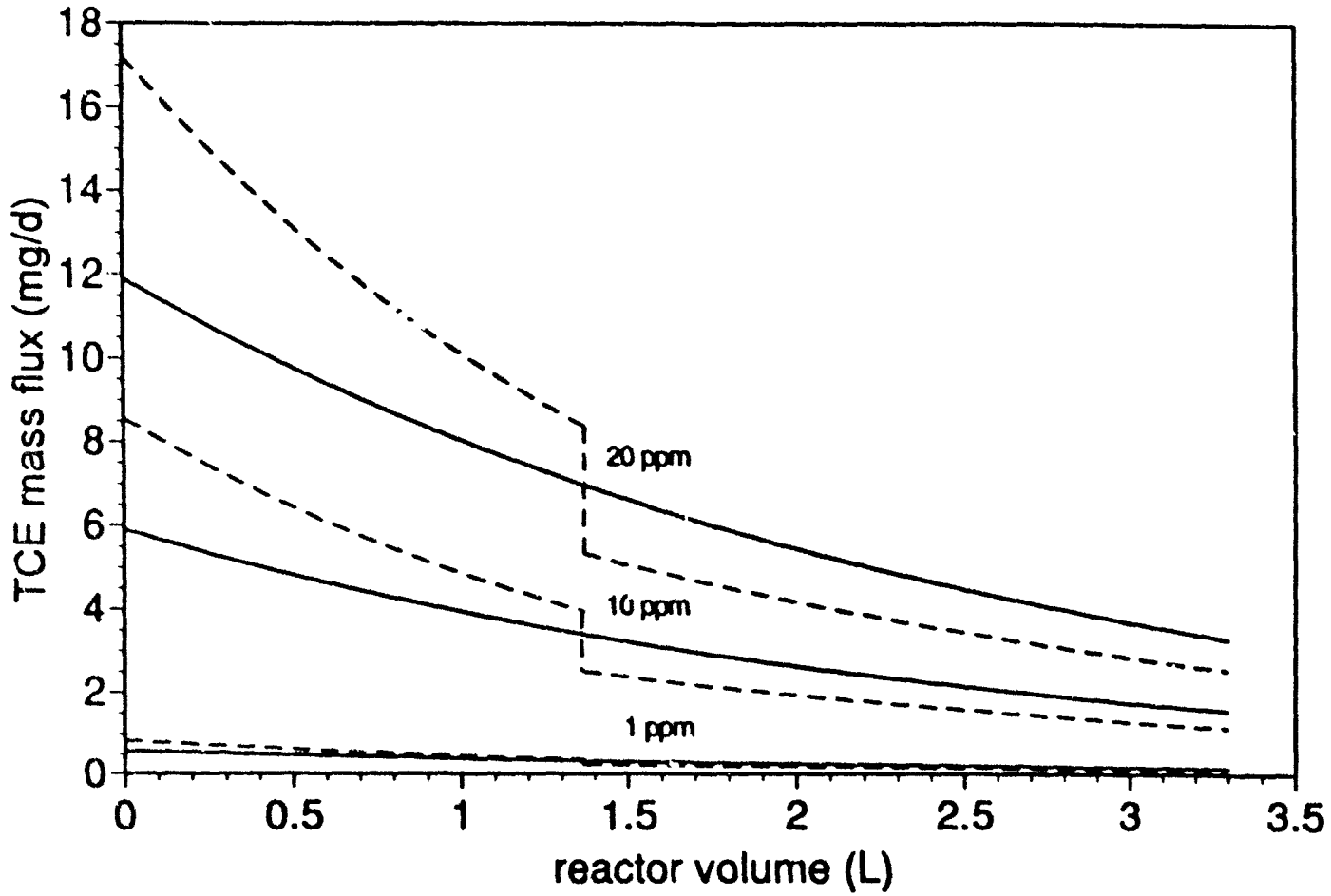

Figure 42. Model comparison of 1 mg/L, 10 mg/L, and 20 mg/L nominal TCE feed concentrations, based on experimental data from single-pass experiments. Dashed lines represent cross-flow conditions and solid lines represent single-pass conditions. Drop in TCE mass flux at 1.368 Liters for cross-flow models represents the effect of dilution due to the feed from CSTR into Column 3.

The initial biomass levels at the top of Column 3, $X_{0,1}$ were computed from a dilution equation:

$$\frac{F_s}{F_s + F_{rec}} X_s + \frac{F_{rec}}{F_s + F_{rec}} 360 mg cells / L = X_{0,1} \qquad (12)$$

where F_s is the total flow rate exiting Column 2 ($F_s = F_{rec} + 2$ mL/min), F_{rec} is the recycle stream from the CSTR entering Column 3, and X_s is the active biomass concentration (mg/L) exiting Column 2 (computed by first run of model). The model was again run for the three nominal TCE feed concentrations in the cross-flow mode, by iterating the model to match q_{rec} with q_{rec} obtained by Equation 10.

The results of the cross-flow operation of the Alvarez-Cohen and McCarty model is also shown in Figure 42. It is apparent that the cross-flow mode is advantageous over the single-pass mode of operation of the bioreactor for the 1 mg/L and 10 mg/L cases by producing lower TCE concentrations exiting Column 4, where the model predicts a 15.3 percent and 5.9 percent lower final TCE flux during the cross-flow mode over the single-pass mode for the 1 mg/L and 10 mg/L, respectively. However, the model predicted a 2.6 percent decrease in final TCE flux for the cross-flow mode during the 20 mg/L case. The increased TCE concentrations, S, for the cross-flow mode over the single-pass mode at the top of Column 1, S_{0_1} and the bottom of Column 2, S_{e_2}, is due to less dilution of the TCE feed during cross-flow operation. During this time the rate of degradation is greater during cross-flow operation for the 1 mg/L and 10 mg/L cases and less for the 20 mg/L case in this portion of the reactor.

Table 8 shows model generated data of TCE mass flux rates, TCE concentrations, and biomass concentrations throughout the plug-flow portion of the bioreactor. The reason for an increased rate of degradation in Columns 1 and 2 during the cross-flow mode for the 1 mg/L and 10 mg/L cases is due to an increased TCE concentration ($S = q / F_T$) of 49.5 percent and 46.7 percent over the single-pass mode at the top of Column 1, S_{0_1}. However, there is also a 14.3 percent decrease in biomass concentration at the top of Column 1, X_{0_1}, during cross-flow operation. The reason why cross-flow operation is less efficient for the 20 mg/L case is attributed to the fact that the extent of loss of biomass activity is proportional to S, and for the 20 mg/L case, where S is large compared to the 1 mg/L and 10 mg/L cases, a larger percentage of the biomass is deactivated during cross-flow operation, and the kinetic advantage of a higher TCE concentration is lost. Based on Alvarez-Cohen and McCarty's model, the expense of the increased rate of degradation, due to large S, is an increased loss of biomass exiting Columns 2 and 4. This point was reinforced by a sensitivity analysis of the model on biomass with the three TCE feed concentrations examined (Table 8).

The model was also examined for CSTR biomass concentrations of 180 mg/L and 720 mg/L for the 1 mg/L, 10 mg/L, and 20 mg/L cases, in order to look for a decreased final TCE mass flux during cross-flow operation with a CSTR biomass concentration of 180 mg/L and an increased final TCE mass flux during cross-flow operation with a CSTR biomass concentration of 720 mg/L. Figure 43 shows the results for the 1 mg/L case, which demonstrates that as the CSTR biomass concentrations

TABLE 8. MODEL-GENERATED TCE FLUXES AND BIOMASS CONCENTRATIONS DETERMINED FOR REPRESENTATIVE INLET TCE CONCENTRATIONS.

		1 mg/L *single-pass*	10 mg/L *single-pass*	20 mg/L *single-pass*	1 mg/L *cross-flow*	10 mg/L *cross-flow*	20 mg/L *cross-flow*
q_{0_m}	*mg/d*	3.70	39.57	86.86	3.23	33.9	72.62
q_{1_m}	*mg/d*	2.13	24.54	58.03	1.45	18.00	44.55
q_{2_m}	*mg/d*	2.13	24.54	58.03	1.80	23.10	59.57
q_{3_m}	*mg/d*	0.98	12.92	35.11	0.83	12.16	36.04
X_{0_m}	*mg/L*	300	300	300	257	257	257
X_{1_m}	*mg/L*	297	272	245	251	205	166
X_{2_m}	*mg/L*	297	272	245	296	270	247
X_{3_m}	*mg/L*	295	250	202	294	249	203
S_{0_m}	*mg/L*	0.214	2.29	5.03	0.320	3.36	7.20
S_{1_m}	*mg/L*	0.123	1.42	3.36	0.144	1.79	4.42
S_{2_m}	*mg/L*	0.123	1.42	3.36	0.104	1.34	3.45
S_{3_m}	*mg/L*	0.057	0.748	2.03	0.048	0.704	2.09

decrease, the percent difference of effluent TCE mass fluxes exiting Column 4 decreases. This relative difference is expressed as:

$$\frac{q_{e,1,SP} - q_{e,1,CF}}{q_{e,1,CF}} = \%diff \qquad (13)$$

This effect is also shown for the 10 mg/L case in Figure 44, where the percent diff becomes negative with a CSTR biomass concentration of 180 mg/L. Again, this is due to a larger fraction of inactivated biomass due to a larger S with a lower X_{0_m} during cross-flow operation. The positive percent diff for the CSTR biomass concentration cases of 720 mg/L and 360 mg/L is also due to a longer residence time in Columns 1 and 2 during cross-flow operation from the lower F_{T_0} (10.08 L/d for cross-flow operation vs. 17.28 L/d for single-pass operation). The decrease in percent diff with a decrease in the CSTR biomass concentration is also shown in Figure 45 for the 20 mg/L case.

In order to predict when cross-flow operation is beneficial over single-pass operation, the percent diff was plotted for each TCE feed concentration vs. CSTR biomass concentration (Figure 46). At those points on the line above percent diff = 0, cross-flow operation is beneficial, and below the percent diff = 0, single-pass operation is favorable. Figure 47 is a plot of percent diff = 0 vs. TCE feed concentration, and allows a prediction of the favorable mode of operation for the bioreactor, based on CSTR biomass concentrations and TCE feed concentrations.

The results of the Alvarez-Cohen and McCarty model are consistent with the increased removal percentages obtained experimentally during the cross-flow operation of the bioreactor over the single-pass operation. It is apparent from the model that increasing biomass concentrations will increase the rate of TCE degradation and the removal percentage of TCE. During experimental operation of the bioreactor, the dewatering device/cell separator was inefficient in maintaining high levels of biomass in the reactor. By implementing a more effective cell separator, which would increase the biomass levels throughout the bioreactor, greater rates of TCE removal may be obtained (hence, greater TCE removal percentages). The model demonstrates that by adjusting residence times and TCE concentrations throughout the reactor, the efficiency of the bioreactor may be maximized.

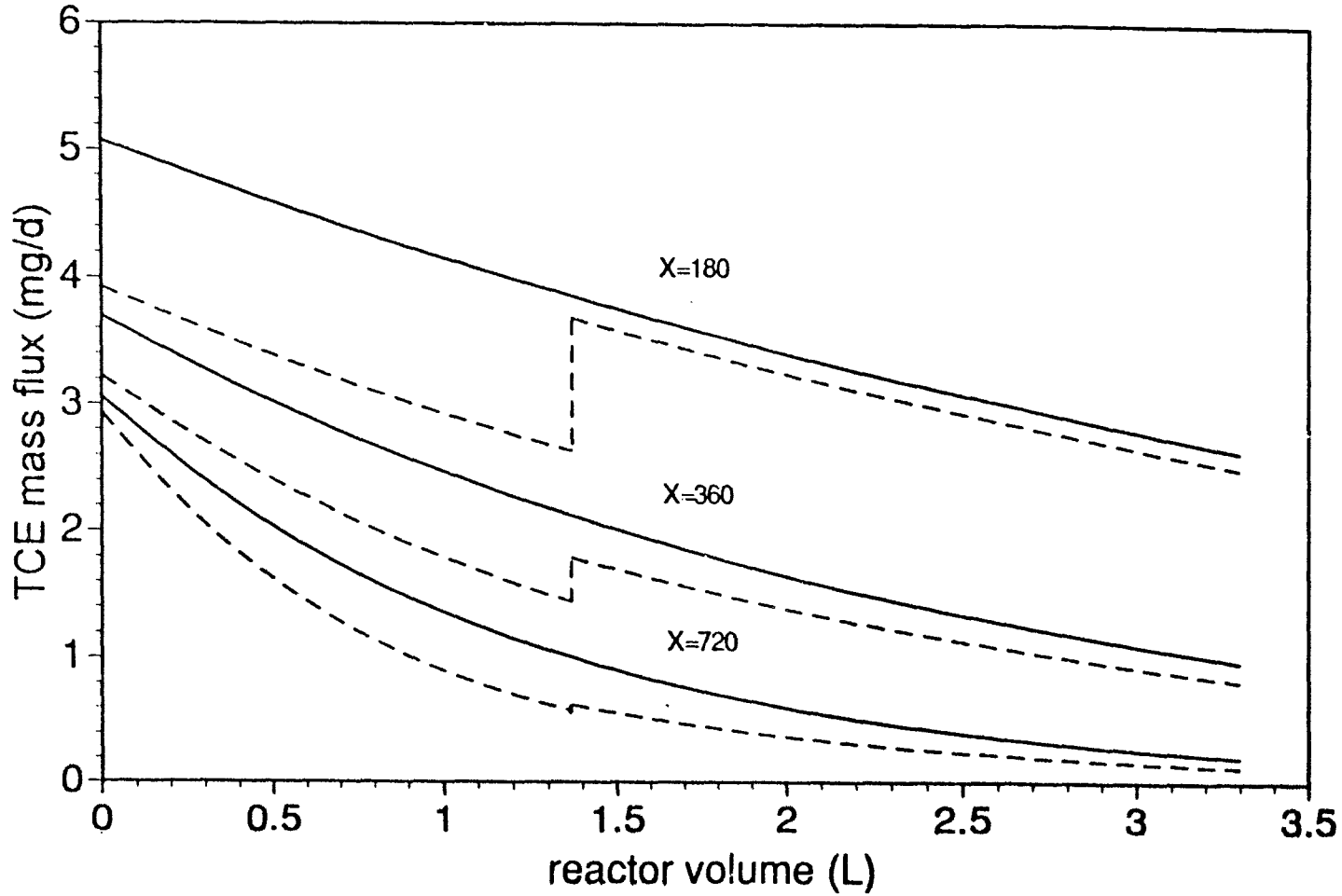

Figure 43. Model comparison for 1 mg/L TCE feed with varying CSTR biomass concentrations of 180 mg/L, 360 mg/L, and 720 mg/L in single-pass (solid line) and cross-flow (dashed line) modes.

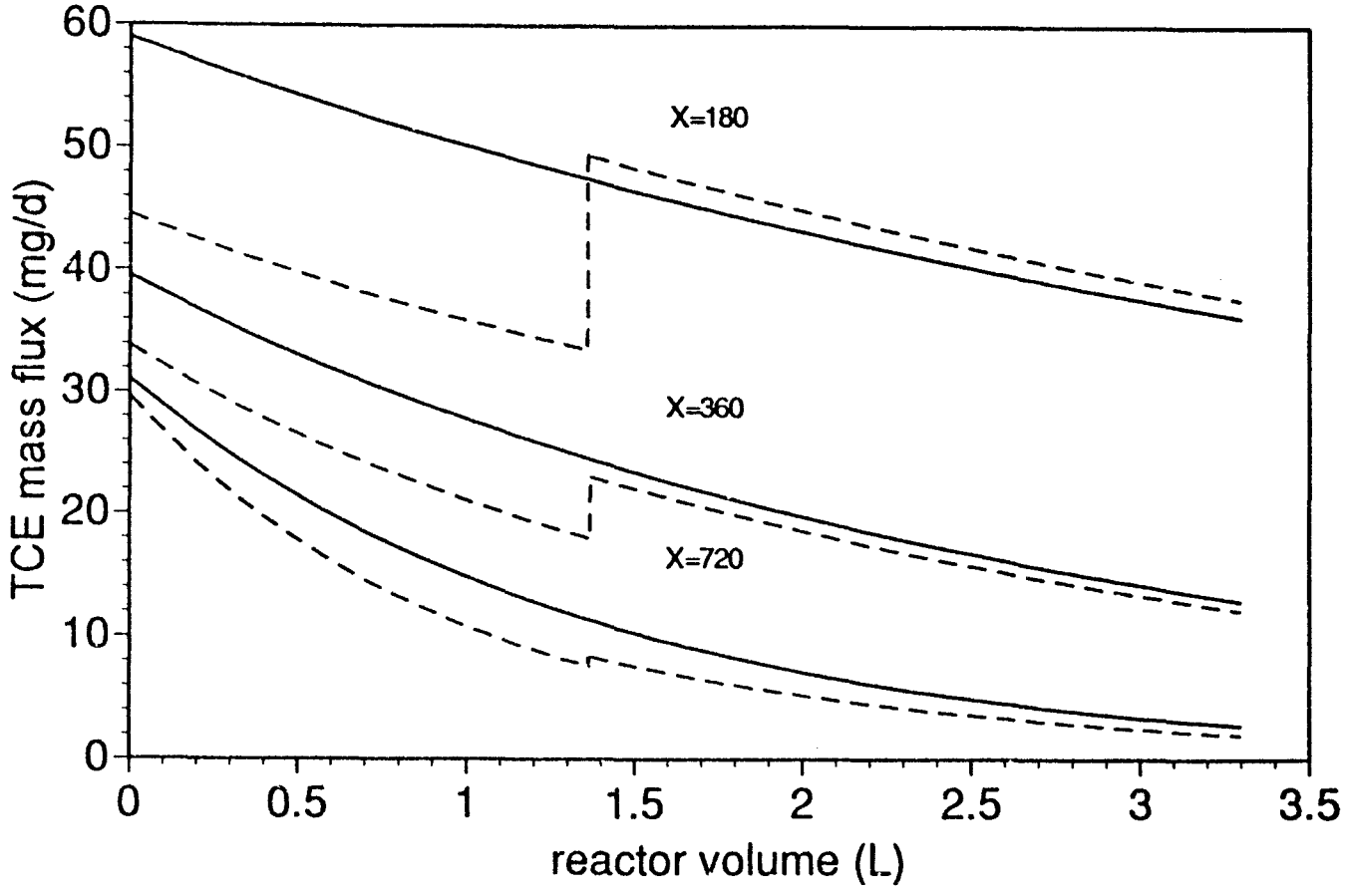

Figure 44. Model comparison for 10 mg/L TCE feed with varying CSTR biomass concentrations of 180 mg/L, 360 mg/L, and 720 mg/L in single-pass (solid line) and cross-flow (dashed line) modes.

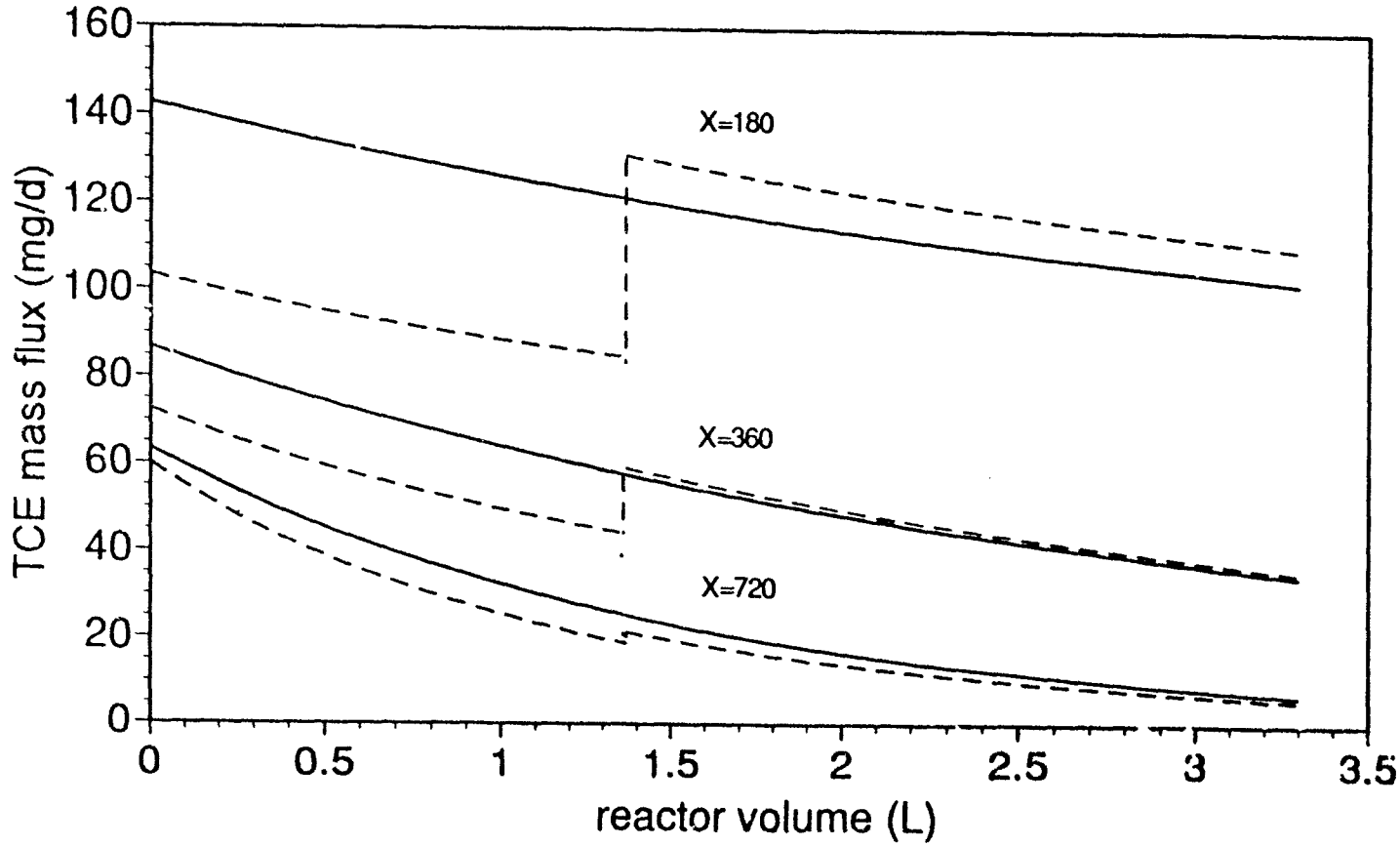

Figure 45. Mode comparison for 20 mg/L TCE feed with varying CSTR biomass concentrations of 180 mg/L, 360 mg/L, and 720 mg/L in single-pass (solid line) and cross-flow (dashed-line) modes.

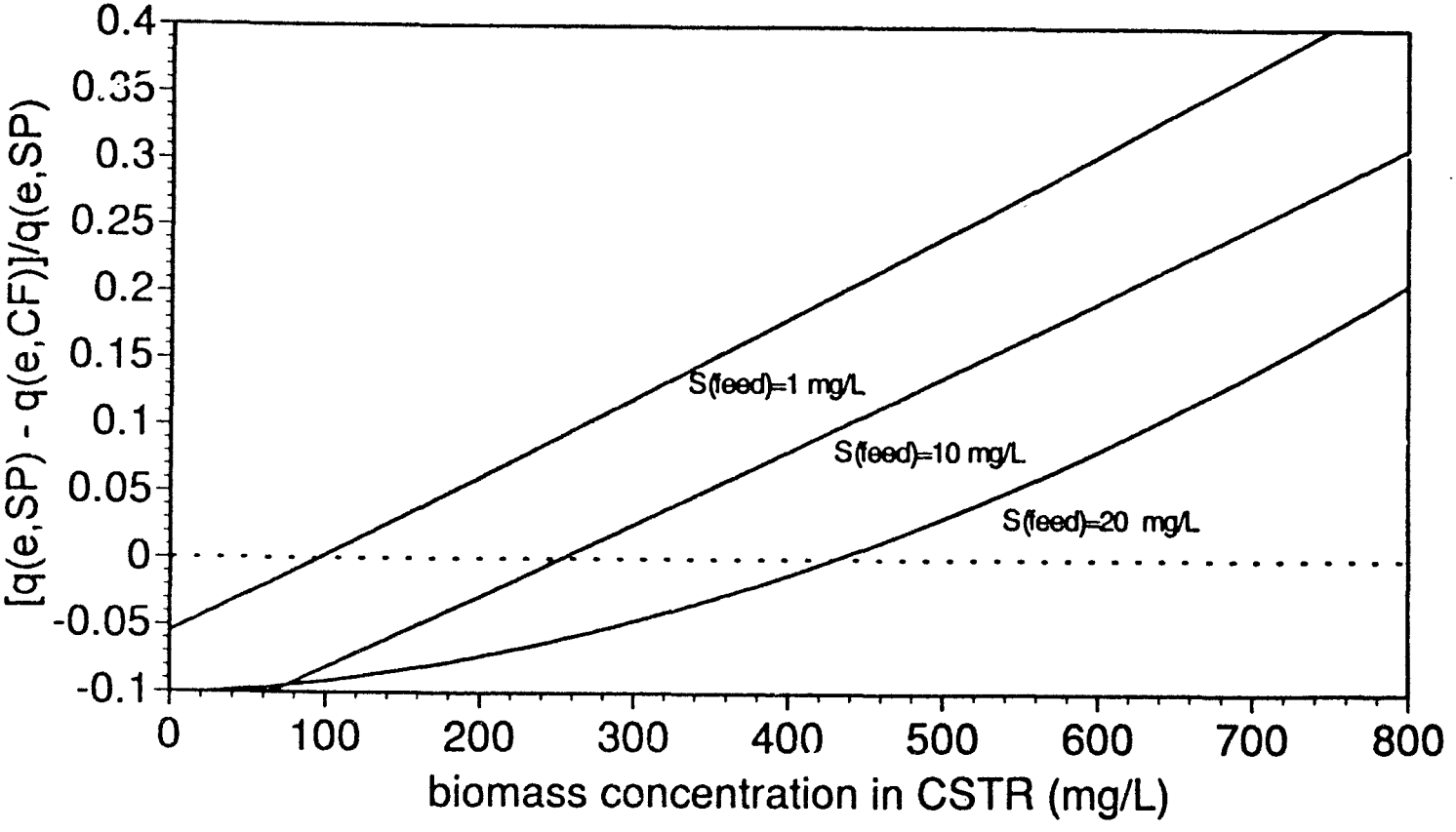

Figure 46. Fractional effluent differences between single-pass and cross-flow operating modes with varying biomass concentrations for 1, 10, and 20 mg/L TCE feeds.

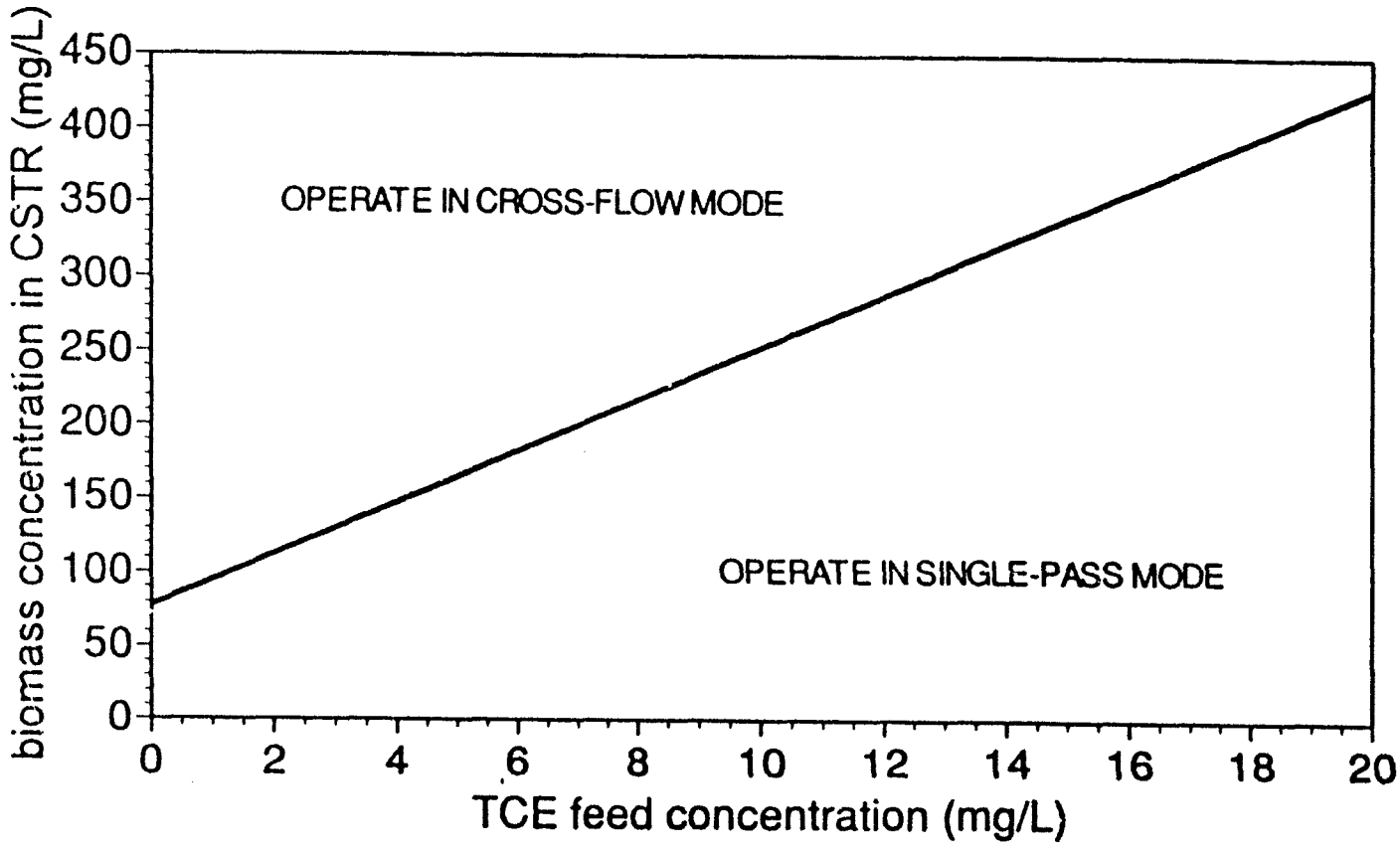

Figure 47. Cross-flow/single-pass operating mode boundary as a function of CSTR biomass concentration and TCE feed concentration. Area above line indicates region where cross-flow is advantageous; area below line favors single-pass mode.

SECTION VI

PROCESS SCALE-UP

The experimental bench-scale unit used in this research (Section IV) has been scaled up from 2 mL/min. to 0.5 gallon-per-minute (1.9 L/min), based solely on the hydraulic residence time (i.e., a scaleup factor of 946). No attempt at optimization of the unit was possible because an accurate mathematical model of the biological process and process variables do not exist at this time. The base-design influent TCE concentration is 1 mg/L. Results expected from the pilot-scale unit should be similar to those obtained from the bench-scale unit if operated under similar conditions. No provisions have been made for temperature control.

Equipment required for the pilot-scale unit is summarized in Table 9. Standard tubing and connections can be used or the unit could be piped. Tubing is simply more convenient, either 3/8" or 1/2" is feasible. The 1/2" tubing more closely represents the conditions of the bench scale unit, while the 3/8" tubing will give flow velocities of ≈3 ft/sec giving better suspension of the culture. The four plug flow columns should be stacked two and two instead of putting all four together like the bench-scale unit to minimize the height of the scaled-up unit. A second 2.5 gallons-per-minute pump will be required between the second and third columns to accommodate this arrangement.

The fermentor and agitator are the major cost items for this unit. It is probably more cost-effective to purchase the unit as a package from a vendor like New Brunswick. These units are normally skid mounted and can be equipped with pH and temperature control. If the main fermentor is temperature-controlled this is probably all that would be required in a temperate climate. There is no reason not to operate the unit liquid full; this will eliminate some mass balance problems with TCE and reduce the overall size and expense of the fermentation unit. The reactor should be rated for at least 50 psig for operating purposes. The bench-scale unit did not require baffling, however, the 600-gallon unit should have 2 - 4 inch baffles on the sides for improved mixing. Reproduction of the agitation is a problem; the bench-scale unit had a very high and unknown power input. The power input is unknown because it is a function of the agitator rpm which was on a variable speed controller and thus varied throughout the experimental period. A 50-hp input is well above that normally found in a fermentor but should represent the lower end of the bench-scale unit's power input.

Figure 48 gives the process flow diagram. Pump P-2 takes fresh cells from the CSTR and sends 2.5 g to the first plug flow reactor, C-1 for contacting with the TCE stream. The remainder of the flow from P-2 is sent to the Cell Separator where 0.5 gallon-per-minute of purified water is removed from the system, with the concentrated cell stream being sent back to the CSTR. A flow of 0.5 gallon-per-minute of TCE-contaminated water is sent to the first contacting column using metering pump P-1. The combined TCE/cell stream flows through the four contacting plug flow reactors and back to the CSTR. Pumps P-3, P-4 and P-5 have been included to insure a uniform flow of cells. These pumps are probably unnecessary as long as the settling velocity of the cells is not large. Methane and oxygen enter the system on flow control and are sparged into the bottom of the CSTR. Excess gas is vented from the CSTR on pressure control using a back pressure regulator or pressure control valve.

The unit's performance depends on the culture density and activity. Both of these quantities varied a great deal during the bench-scale experiments. No attempt has been made to alter the ratio of residence times between the fermentor and plug flow reactors, nor to optimize the residence times. In order to optimize the unit, a mathematical model which takes into account, culture density, sMMO activity, TCE concentration, TCE biodegradation rate, and TCE toxicity toward both the culture and the enzyme is required and presently not available.

Because of the small scale of the bench-scale unit the cell-separator was ineffective, resulting in substantial loss of cell culture. It is estimated that culture densities of 10 to 20 times greater than those used in the bench-scale experiments are feasible. The effect of cell density on the rate of TCE degradation appears to be linear. A 10-fold increase in the cell density would thus result in converting the 0.5 gallon-per-minute pilot plant into a 5.0 gallon-per-minute unit, assuming the process pumps and lines along with the methane and oxygen feed lines were appropriately enhanced.

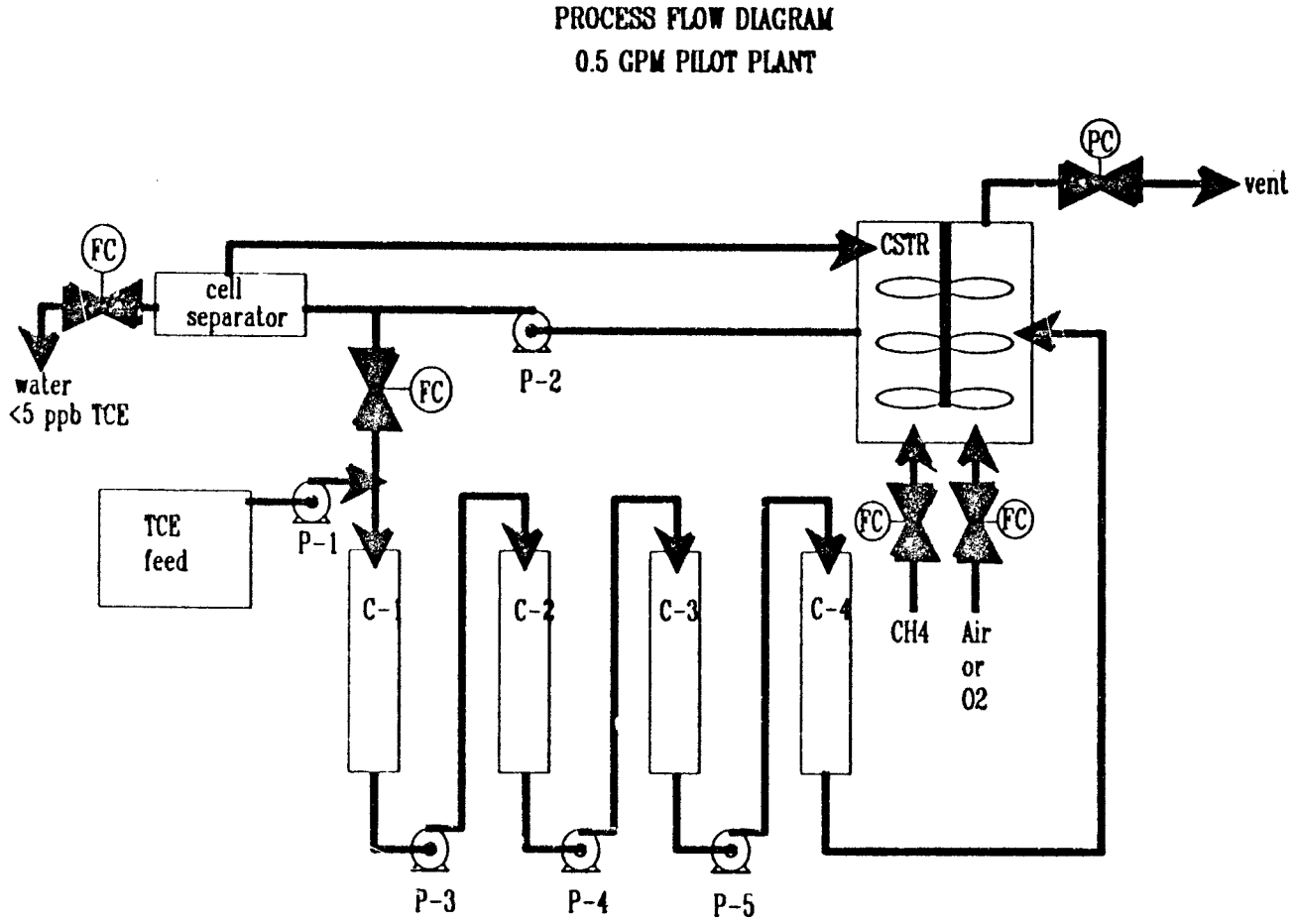

Figure 48. Process flow diagram of 0.5 gallon-per-minute pilot-scale bioreactor system based on scaleup of bench-scale unit.

TABLE 9. EQUIPMENT LIST FOR 0.5 GALLON-PER-MINUTE PILOT-SCALE BIOREACTOR

Component	Specifications
Tubing:	1/2" or 3/8" stainless steel
Valves:	12 1/2" or 3/8" ball valves, stainless steel
Fermentor:	600 gal., stainless steel, 40 psig rating, liquid full with pressure control from a backpressure regulator
Flow Controllers:	2 with a 0.4 ft^3/sec capacity 1 with 0.5 gallon-per-minute liquid flow 1 with 2.5 gallon-per-minute liquid flow
Plug Flow Bioreactors:	4-150 gal. cylinders, 1.5 ft diameter and 11.5 ft long, stainless steel, 200 psig rating
Agitators:	2 downflow turbines and 1 radial flow turbine with a total power input of ≈50 hp, with a mechanical pressure seal of 50 psig
Pumps:	1 0.5 gallon-per-minute centrifugal capable of delivering 20 psi of head, stainless steel construction
	3 2.5 gallons-per-minute centrifugal capable of delivering 20 psi of head, stainless steel construction
	1 5.0 gallons-per-minute centrifugal capable of delivering 20 psi of head, stainless construction
Centrifuge:	0.5 gallon-per-minute, Sharples or Bird continuous solid bowl, sized by manufacture for separating specific culture to be used from water, stainless steel construction
Backpressure Regulator:	0 - 50 psig rating, stainless steel construction
Dissolved Oxygen Probes:	3 Ingold, 3/4" autoclavable pressure probe

SECTION VII

CONCLUSIONS

1. A recirculating pressurized two-stage bioreactor system has been developed at the bench scale and successfully operated for methanotrophic co-metabolic oxidation of TCE at feed solution concentrations ranging from 0.2 mg/L to 20 mg/L. The bioreactor system has demonstrated the effectiveness of separation of the co-metabolic TCE oxidation stage from that of biomass maintenance and growth.

2. The critical factor for maintaining efficient TCE degradation has been demonstrated to be the level of active microbial biomass that contacts TCE in a non-methane competition mode. Maximum operating efficiency for TCE removal was not achieved during the bioreactor operational period due to mechanical failures of the system dewatering devices that allowed greater than 60 percent of the biomass to escape the system. However, even under these suboptimal conditions, treatment effectiveness was enhanced by extending the cell-TCE contacting time in additional TCE contacting columns.

3. System operating characteristics during cross-flow operation, in which fresh cells are introduced into the TCE-containing stream, improved TCE removal capacity significantly (i.e. reduced effluent TCE levels) over the performance obtained when cells were added only at the beginning of the contacting stage. Performance enhancement increased at higher TCE concentrations. Results were consistent with mathematical modeling of the system that incorporated inactivation of TCE degradation by TCE. Existing mathematical models appear to be adequate for describing and predicting TCE removal kinetics in the two-stage bioreactor system.

4. Soluble methane monooxygenase (sMMO) activity was proved to be stable and robust, and inactivation of the enzyme was shown to be largely recoverable by addition of formate, presumably by enhancing synthesis of new enzyme.

SECTION VIII

RECOMMENDATIONS

Successful performance of the two-stage bioreactor technology in this project should lead into additional tests to demonstrate the effectiveness of the design in groundwater treatment applications. Because the biomass level was identified as a key variable, the present bioreactor unit should be modified to increase the effectiveness of biomass retention. Feedback controls should be installed on the system to ensure maintenance of operational parameters at optimal levels.

Operational tests should continue with the bench-scale unit presently available. Tests to evaluate effects of additional parameters should be completed in order to optimize operating conditions. The promising initial results of formate addition in enhancing recovery of sMMO following TCE treatment should be continued to optimize its effectiveness. In addition, preliminary results indicating similar effectiveness of other compounds should lead to tests in the bench-scale system. Test length should be increased to evaluate the long-term stability of the system. These tests should include using actual TCE-contaminated groundwater obtained from Air Force sites. A preliminary evaluation of the system's economics should be conducted to identify the operating variables that are most important in cost minimization.

Bench-scale results are already sufficiently promising to warrant plans to scale up the bioreactor design that was tested in this project for engineering evaluation. The 0.5 gallon-per-minute scale is recommended as the largest that could be developed on the basis of the performance of the present unit. However, additional tests, using the bench-scale unit, are needed to optimize flow rates and minimize reactor volumes. Additional engineering design would then be required prior to construction of a pilot unit to optimize reactor configuration.

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